

**Comparative Immunochemical Studies
on Normal and Monoclonal
Immunoglobulin M**

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LIST OF ABBREVIATIONS

- BAEE : N - benzoyl - L - arginine ethyl ester
B-lymphocyte : Bone-marrow derived lymphocyte
BIS : N,N' - methylenediacrylamide
CD : Circular dichroism
C-region : Constant region
DTT : Dithiothreitol
FAC : Fast anodal component
GuHCl: Guanidine hydrochloride
IE : Immunoelectrophoresis
M.W. : Molecular weight
ORD : Optical rotatory dispersion
PEG : Polyethylene glycol
PMSF : Phenylmethylsulphonylfluoride
SDS : Sodium dodecyl sulphate
SDS-PAGE : Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED : N,N,N',N', -Tetramethylethylenediamine
T-lymphocyte : Thymus derived lymphocyte
V-region : Variable region

The abbreviations used for the immunoglobulins and their sub-units and fragments are in accordance with the recommendations of the World Health Organisation (Bull. World Health Organ., 30, 477, 1964).

The abbreviations used for the complement system are in accordance with the recommendations of the World Health Organisation (Immunochimistry, 7, 137, 1970).

Chapter 1

INTRODUCTION

"One of the most exciting problems in molecular biology today is the elucidation of the relationship between the biological properties of immunoglobulins and their organisation at the several levels of protein structure."

(Dorrington and Tanford, 1970.)

The first report relating the physico-chemical properties and the biological activity of immunoglobulins appeared more than thirty years ago when Tiselius and Kabat (1939) reported that antibody activity was associated with proteins having a γ -globulin electrophoretic mobility. The first significant investigation of the immunoglobulins at a submolecular level was undertaken by Porter (1959) and subsequently led to the publication of the entire amino acid sequence of Immunoglobulin G (Edelman et al., 1969). The impact which this pioneering work had on our understanding of the intricate interaction between antibody and antigen, and therefore on immunology in general, was acknowledged in 1972 when Porter and Edelman were awarded the Nobel Prize for Medicine.

Although the G class of immunoglobulins was the first to be associated with antibody activity, immunoglobulin M (IgM) had been recognised earlier in the sera of cattle and horses (Heidelberger and Pederson, 1937). However, initial attempts at the structural elucidation of this very large protein molecule were delayed until

Deutsch and Morton (1957) showed that these immunoglobulins were dependent on disulphide bridges for the integrity of their polymeric structure. In a series of three papers Miller and Metzger (1965a, 1965b, 1966) gave proof of the pentameric structure of IgM, and also showed that it probably existed as a circularly arranged molecule consisting of subunits analogous to IgG, each subunit being built up of two heavy (H) and two light (L) chains, covalently linked (see Fig. 7).

The subsequent discoveries of Immunoglobulin A (IgA) (Grabar et al., 1956), Immunoglobulin D (IgD) (Rowe and Fahey, 1965) and Immunoglobulin E (IgE) (Ishizaka et al., 1966b) completed the list of identified immunoglobulin classes known to occur naturally in human serum. The basic difference between these immunoglobulin classes lies in the amino acid sequence of their H-chains, the L-chains being one of two types (Kappa or Lambda) for all immunoglobulins. These differences are reflected mainly in the biological activities, other than antigen binding, of the different immunoglobulin classes. Some of the properties imparted to these molecules by the nature of their respective H-chains, are the predominant appearance of IgA in secretions like saliva, tears and milk (Tomasi et al., 1965) and of IgM, probably because of its size, almost exclusively in the intravascular pool (Barth et al., 1964). Nowhere, however, are these class differences better reflected than in the differential activation of the cytolytic complement system.

The typical sequence of events in the cytolytic process begins with the attachment of C1 (consisting of a Ca^{++} bound complex of C1q, C1r and C1s) to the cell surface to form an active C1-immunoglobulin

complex (Fig.1). Activated C1 then mediates the bindings of C4 and C2 to the cell which in turn forms a short-lived complex responsible for the activation of the "amplifier molecule" of the system, C3 (Figs. 2-4). Other components in the chain of reactions are C5, C6, C7, C8 and C9 and these are sequentially activated by the component or complex of components preceding it. C9 is ultimately responsible for rapidly lysing the foreign cell. The trigger in this sequence, therefore, is the binding of C1q to immunoglobulins, and this is determined by the amino acid sequence of the H-chains. Thus IgA is unable to bind complement whereas IgM and the IgG1, IgG2 and IgG3 subclasses can do so efficiently (Müller-Eberhard, 1969).

Although it is known that amino acid sequence to a great extent determines the conformation of proteins (Lumry and Eyring, 1954; Crick, 1958; Anfinsen, 1962) and hence must play an essential role in the three dimensional structure of these molecules, it is today accepted that all the immunoglobulin classes have a similar gross conformation, namely the Y-shaped molecular model, with "compact domains" as proposed for IgG by Edelman and Gall (1969) and verified visually by electron microscopy (Valentine and Green, 1967).

Perhaps the most significant contribution to our understanding of the relation between immunoglobulin structure and function has come from the amino acid sequence analysis of the L- and H-chains. One of the more important facts that has emerged from these investigations, was that both L- and H-chains could be divided into amino-terminal variable (V) and carboxy-terminal invariable or constant (C) regions (Hiltschmann and Craig, 1965; Lennox and Cohn, 1967) (Fig. 5). Because of the sequence heterogeneity of the V-region, it logically follows that

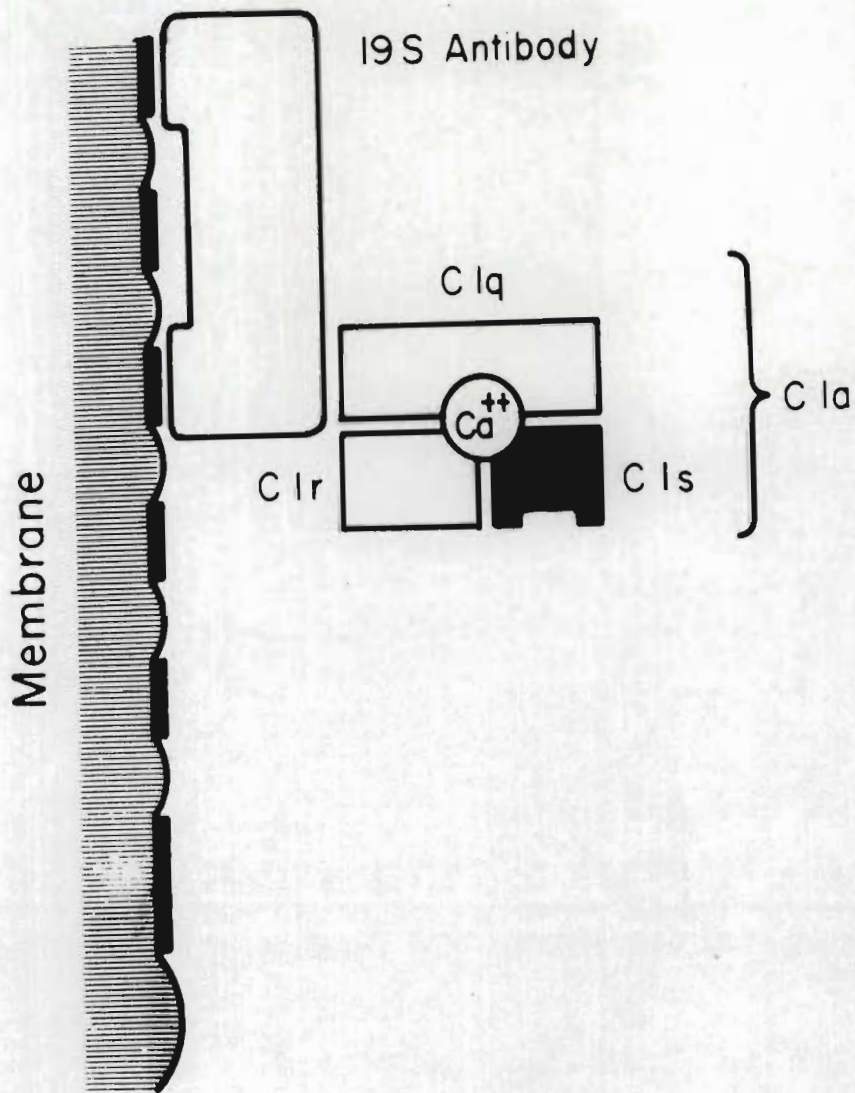


Fig. 1: Schematic illustration of the first component of complement (C1) reacting with a 19S antibody molecule attached to the cell surface. (Taken from Müller-Eberhard et al., 1966).

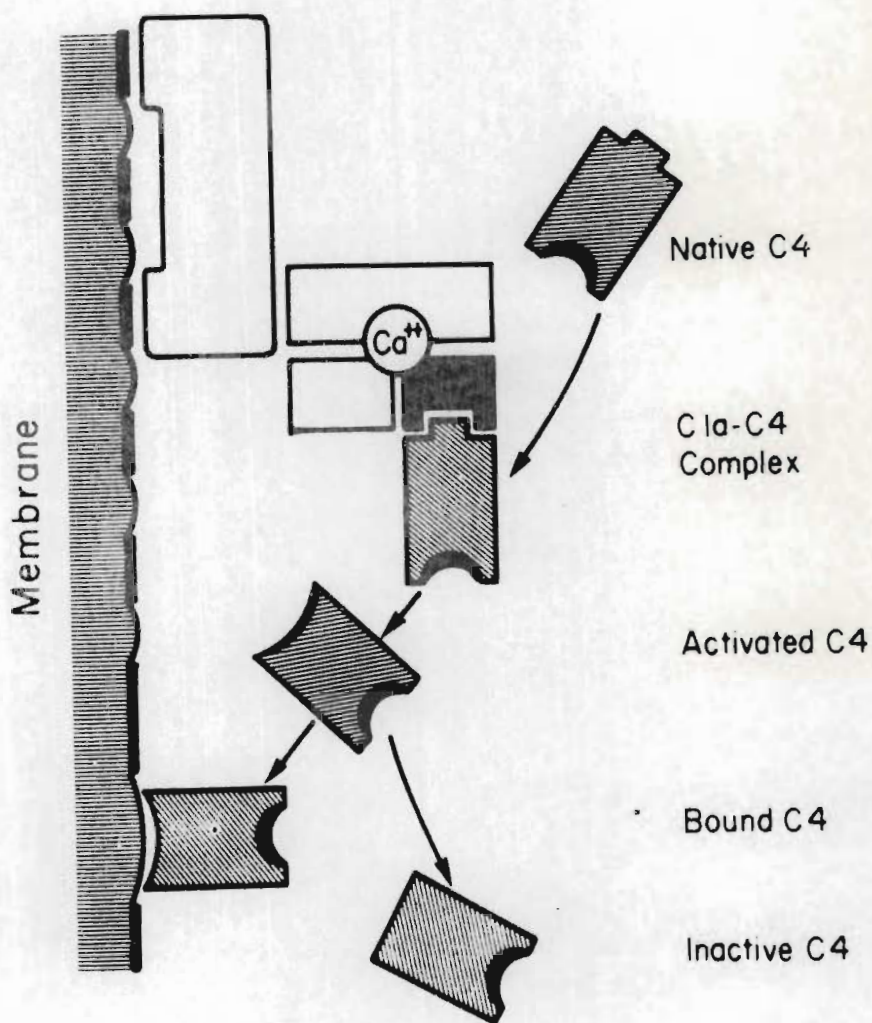


Fig 2: Postulated mechanism of binding of C4 to the surface of a cell under cytolytic attack. (Taken from Müller-Eberhard *et al.*, 1966).

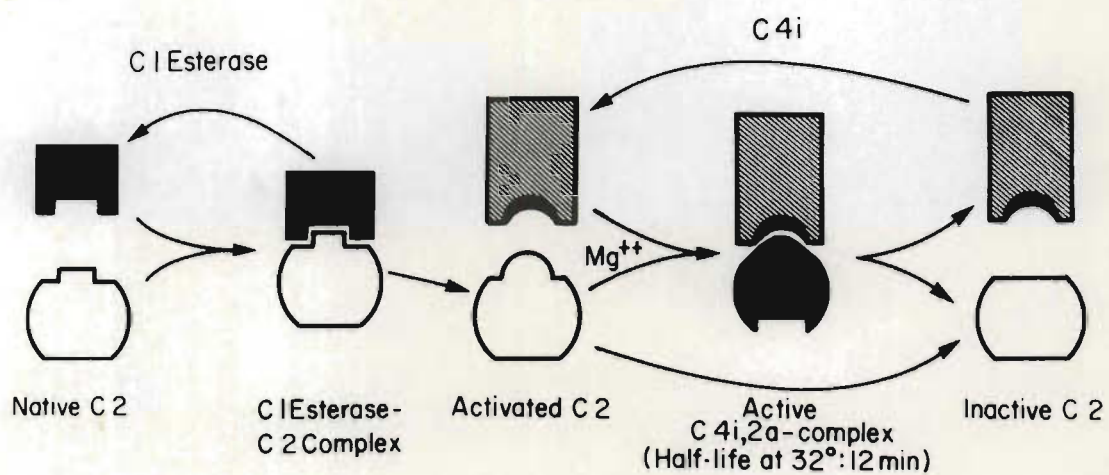


Fig. 3: Illustration of the formation of the short-lived C4C2 complex responsible for activation of C3. (Taken from Müller-Eberhard *et al.*, 1966).

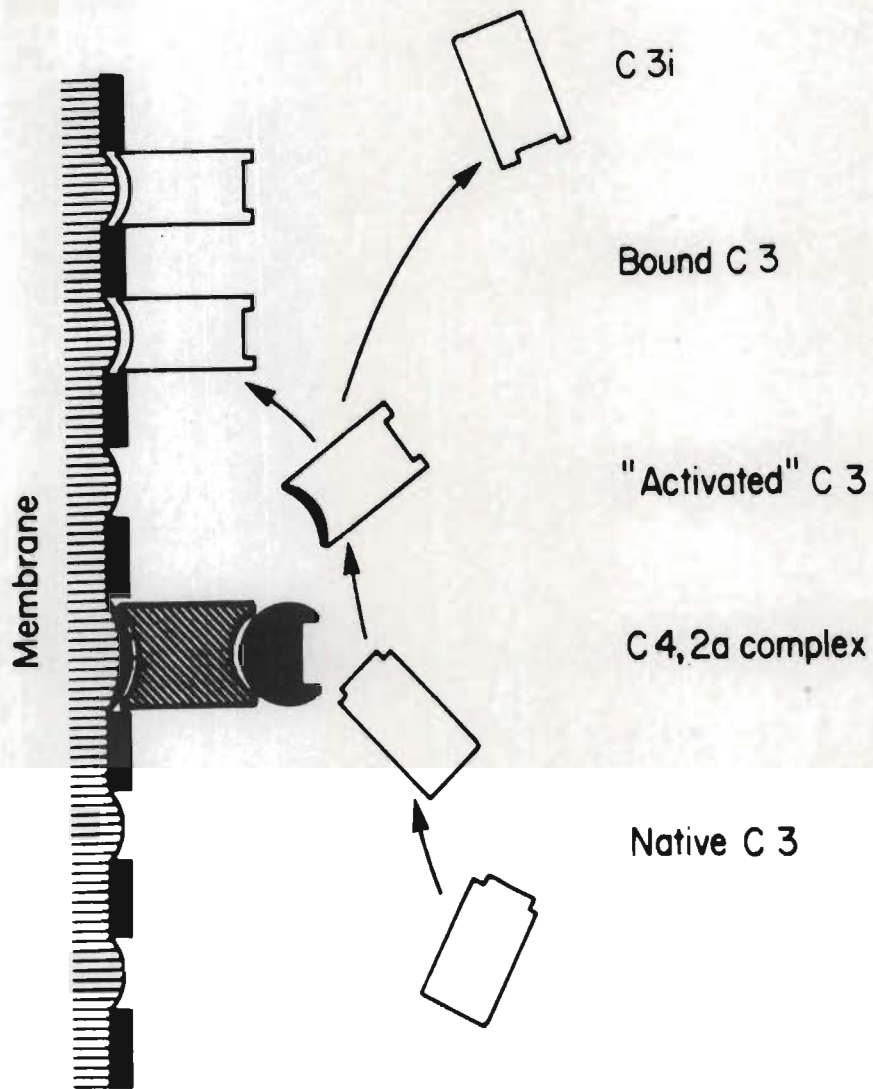


Fig. 4: Mechanism of binding of C3 to the cell surface. (Taken from Müller-Eberhard et al., 1966).

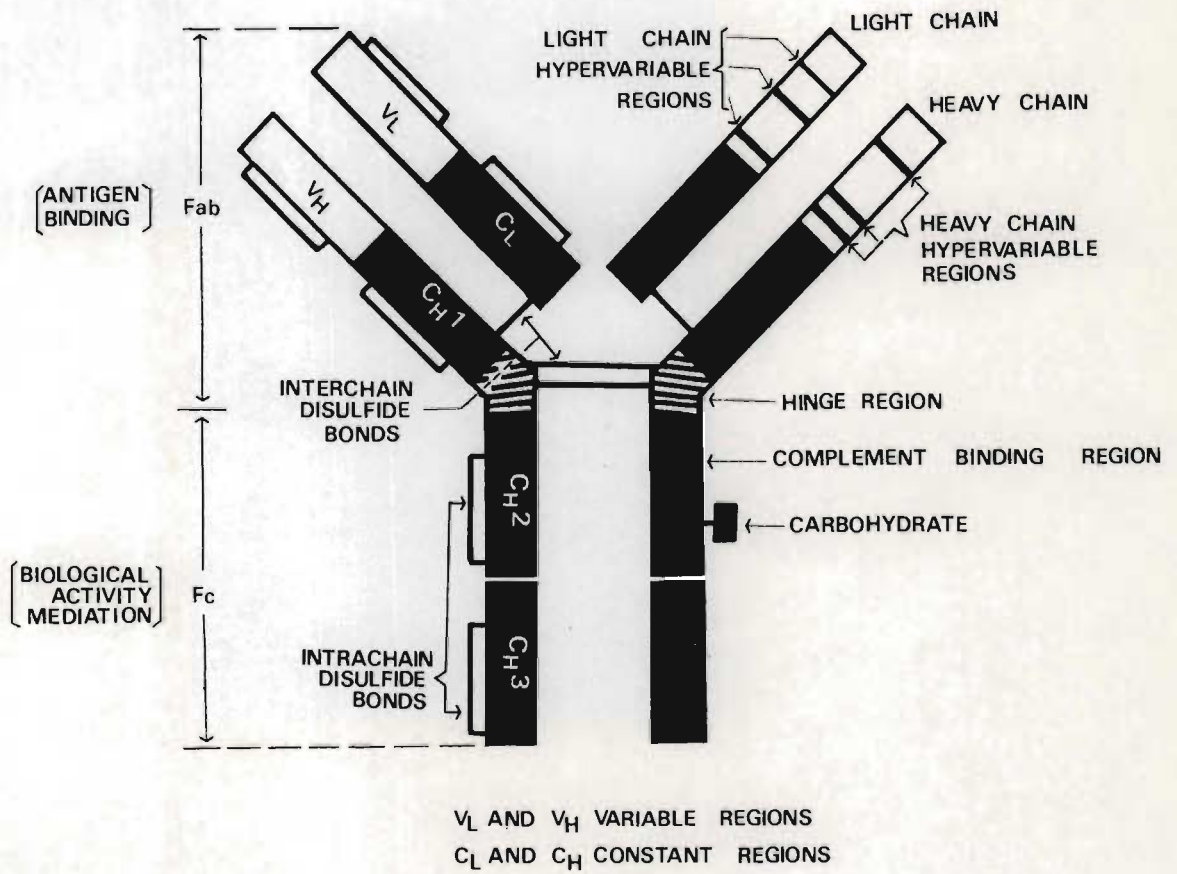


Fig. 5: Diagram of the topology and functional architecture of the IgG molecule. (Taken from Capra and Kehoe, 1972).

it is in fact only the C-region which is responsible for the division of L- and H-chains into different classes and subclasses. Although the V-regions in the L- and H-chains are of about the same length, (110-120 residues) the C-region of the γ -chain is about three times (Edelman et al., 1969) and that of the μ -chain almost four times (Putnam et al., 1972) as long as the V-region of the L-chains. Furthermore, within each C-region of L- and H-chains there exist areas of homology of the amino acid sequence (Hill et al., 1967) and it has been proposed that these originated by duplication of a gene coding for a polypeptide of about 110 residues (Hill et al., 1966; Singer and Doolittle, 1966). These homology regions are thought to coincide with the compact domains (Fig. 5) and it has been postulated (Edelman et al., 1969) that each of these perform at least one biological function such as complete fixation by C_{H2} (Kehoe et al., 1969) or placental transfer.

The occurrence of hypervariable areas (Fig. 5) within the V-regions of L- (Milstein, 1967; Kabat, 1970) and H-chains (Kohler et al., 1970; Capra, 1971) have been postulated and Wu and Kabat (1970) have suggested that these hypervariable areas make direct contact with antigen during the antibody-antigen reaction; that is, they form the antigen-binding site. This seems reasonable, seeing that the areas of lesser variability could be responsible for the overall correct tertiary conformation and the hypervariable regions for the more refined structures necessary for specific binding to the multitude of chemically distinct antigens that may elicit an antibody response. Although these hypervariable areas are shown as being quite far apart in the schematic representation in Fig. 5, they are probably forced into close spatial juxtaposition by the presence of non-covalent interactions, and intra-chain disulphide bridges. The same model applies to both H- and L-chains

and it has been shown by affinity labelling experiments (Thorpe and Singer, 1969; Goetzl and Metzger, 1970; Franek, 1971) that both H- and L-chains do in fact partake in antigen binding. Direct evidence illustrating that only the V-regions of the H- and L-Chains are involved in antigen binding, has recently been obtained by Inbar et al. (1972), when it was shown that the amino-terminal half of the Fab-fragment of a mouse IgA myeloma protein, would bind antigen equally as well as the intact precursor molecule.

As in the case of other proteins, the amino acid sequences of immunoglobulins are genetically determined and, prior to discussing the genetic implications, it might be useful to discuss the origin of the immunoglobulins. Studies conducted at a cellular level are often difficult to interpret due to the lack of parameters that are accurately measurable and there is also a fair amount of confusion in terminology. However, it is generally accepted that complex interactions between various cell types like macrophages, bone marrow (B) and thymus (T) derived lymphocytes and foreign material (e.g. pollen or transplanted tissue) are necessary for subsequent synthesis of antibody molecules complementary to such antigens. The sequence of events during the cellular immunologic response may be briefly outlined as follows: the T-lymphocyte is primed to recognise the antigen and this primed cell, possibly with the assistance of macrophages, transmits a message to the B-lymphocyte stimulating it to proliferate and form a clone of cells producing the appropriate antibody molecules (Fig. 6).

In order to answer the question of how the lymphocyte recognises a specific antigen, it has been postulated that recognition sites exist on its membrane, each of which is structurally complementary to only one antigen. (Mitchison, 1967; Siskind and Benacerraf, 1969). These

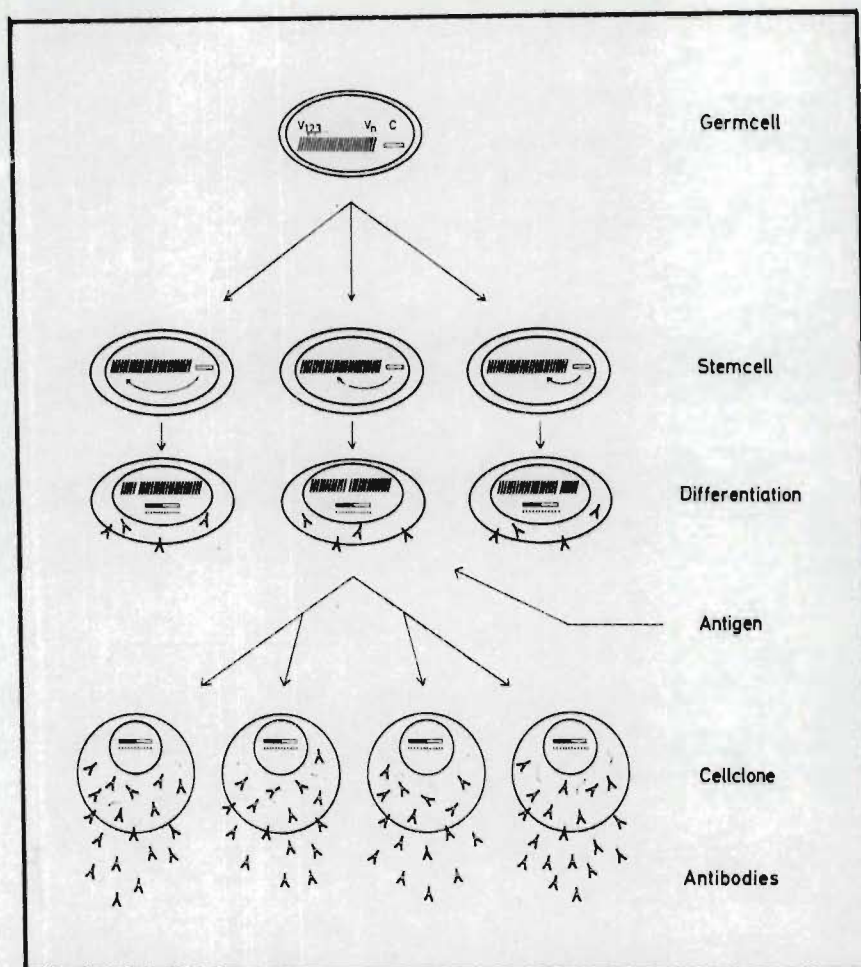


Fig. 6: Antibody formation, a model of cell differentiation. The information of antibody specificity is stored in hundreds of thousands of serially repeated genes for the variable part. This multipotency of the germ-cell is lost during differentiation by fusion of one of these variables (V-) genes with the single constant (C-) gene. This differentiated cell is unipotent: by a normal transcription and translation mechanism it forms antibodies of one specificity. The antigen selects those cells which produce antibodies with the best fitting specificity. These cells proliferate and form an antibody producing cell clone. (Taken from Hilschmann *et al.*, (1970).)

recognition sites are considered to be natural cell-bound antibody molecules or active (i.e. Fab) fragments thereof. The binding of antigen to the lymphocyte through its reaction with this surface receptor, probably initiates the cellular events leading to antibody production. There is ample evidence in the literature indicating the immunoglobulin nature of these receptors (Sell and Gell, 1965; von Furth, 1969; Oppenheim et al., 1969).

The possibility that all V-regions originated from different membrane receptor molecules (Dreyer et al., 1967) and that the V-genes responsible for these proteins later developed a mechanism for joining on to C-genes, is attractive and would explain how the humoral antibodies with their multiple specificities, but relatively few classes, evolved. Since IgM is the first immunoglobulin to appear in ontogeny (Good and Papermaster, 1964; Sterzl and Silverstein, 1967; Uhr and Finkelstein, 1967) and has a special evolutionary position (Grey, 1969) it is perhaps not surprising that it has also been found to be, in some cases, the immunoglobulin class of the lymphocyte receptors (Warner et al., 1970; Dwyer et al., 1972).

The genetic control of antibody synthesis is unique in that there is no other class of mammalian protein where a single peptide chain is under genetic control of two separate genes (Dreyer and Bennett, 1965). Evidence in favour of this hypothesis has been found by Hilschmann et al. (1969), Kohler et al. (1970), Putnam et al. (1972) and Pink et al. (1972) from a comparison of the amino-terminal amino acid sequence of λ , μ - and α -chains. These workers demonstrated that the V-regions associated with different H-chain classes could be subdivided into subgroups (V_{H1} , V_{H11} , V_{H111}) on the basis of their amino acid

sequences. The same situation applies to L-chains. The inference of this finding is that the combining site, which is limited to the V-region of the H-chain, may occur in H-chains of different classes and also that a single V-region may be transferred from one C-region to another. The finding that the initial antibody response to a well-defined antigen is at first expressed as IgM and that this may later be "switched over" to antibodies of the IgG class (Nossal et al., 1965; 1971) lends powerful support to such a hypothesis. The recent finding of Wang et al. (1970), that the amino-terminal amino acid sequence of the γ - and μ -chains, isolated from the immunoglobulins of a patient suffering from a biclonal myeloma, were exactly the same, is also evidence in favour of the one-peptide two-gene theory.

In order to accommodate such a model, it has to be assumed that the genetic control for the immunoglobulins is separate for the V- and C-regions of these molecules: one gene for the C-region and many for the V-regions (Hood et al., 1967). Depending then on the specificity of the receptor immunoglobulin on the lymphocyte involved in an immune response, one only of the many V-regions will be derepressed and will be able to combine with the gene coding for the C-region of a single immunoglobulin class (i.e. C_γ , C_μ , C_α). A schematic representation of this model is given in Fig. 6.

These hypotheses explain how antibodies of different specificities can be elicited by a challenge from any particular antigen. However, if a lymphocyte undergoes a neoplastic transformation and retains its antibody-producing capacity, it may also form a clone and produce antibodies with no, or accidental specificity, in exceedingly large amounts. Originating from a single clone as they do, these immunoglobulins will

all be chemically and structurally the same (monoclonal) and are therefore ideal for chemical and biophysical investigation. These abnormal immunoglobulins are called myeloma proteins or, in the case of IgM, Waldenström macroglobulins. Although experience has shown that extrapolation of such findings to the structure of normal immunoglobulin M is generally valid (Metzger, 1970), unequivocal proof of this identity can be obtained from a direct comparison of various parameters of normal and monoclonal IgM. Since the first observation that a human monoclonal immunoglobulin had rheumatoid activity (Kritzman et al., 1961) a growing number of myeloma proteins of human and animal origin have been found to react with a variety of antigenic determinants; that these abnormal proteins can act biologically as antibodies is therefore established. Yet Krause (1970) states "Despite such success, in all of this there is a nagging reservation. Is a myeloma protein, the product of malignant cells, a genuine representative of an antibody?" One of the main purposes of this thesis was therefore to conduct a comparative physico-chemical investigation on normal IgM and a Waldenström IgM and their various subfragments and component polypeptide chains.

At present the world price of fresh plasma is approximately U.S. \$26 per litre. Plasma is therefore a relatively expensive starting material from which to isolate normal IgM for research purposes, especially if the low concentration of this immunoglobulin of 39-117 mg/100 ml serum (Schultze and Heremans, (1966) is taken into account. Donated blood is usually stored at 4-10°C for a maximum period of 21 days and is then regarded as unsuitable for transfusion purposes. Plasma recovered from such outdated blood can be used directly for the isolation of IgM, but since it also contains large quantities of other therapeutically useful products (e.g. IgG, albumin) with greater econ-

omic significance than IgM, the plasma is usually fractionated with the main objective being to recover these latter products. The fractionation procedure employed at the Natal Institute of Immunology is a modification by Kistler and Nitschmann, (1962) of Cohn's method 10 and yields five different protein product fractions. Of these, only fraction I (fibrinogen), fraction II (IgG) and fraction V (albumin) are utilised and fractions III and IV are discarded. However, fraction III is known to contain the bulk of the macroglobulins (Kistler and Nitschmann, (1962)) and it was therefore decided to attempt the purification of IgM from this "waste product".

Because the main purpose of this investigation was to compare the biophysical characteristics of normal IgM and its subfragments and peptide chains with published data (summarised in Fig. 7) that originates from abnormal, monoclonal IgM, the ready availability of macroglobulinaemic plasma was fortuitous. Monoclonal IgM could be isolated in relatively large amounts and direct comparative studies were therefore possible. An added advantage was that monoclonal IgM, rather than the scarcer normal IgM, could be used in preliminary tests aimed at overcoming technical problems of chromatography, tryptic digestion, ultracentrifugation and some others encountered at various times.

The high molecular weight of IgM relative to the other plasma proteins suggests its easy isolation, and molecular exclusion chromatography was used as the first step in the purification of both normal and monoclonal IgM. Whereas the monoclonal IgM could be isolated in pure form by precipitation and column chromatography, normal IgM was more difficult to obtain in immunogenically pure form and various immunoabsorbents had to be developed for this purpose. These procedures are described in

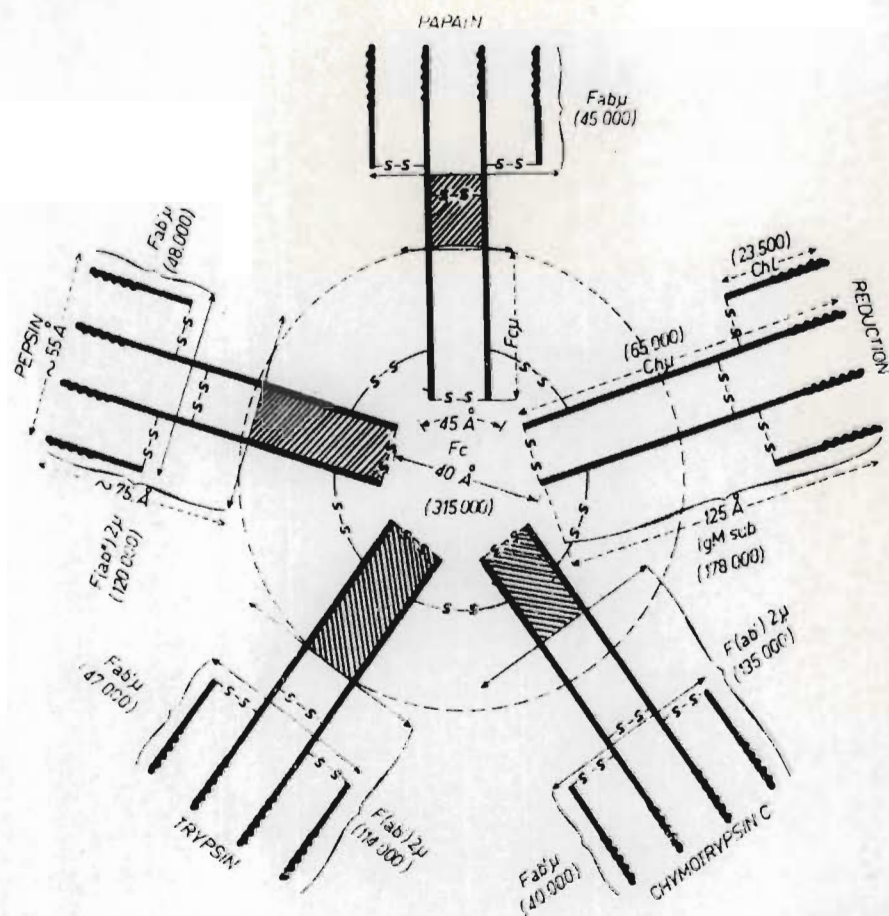


Fig. 7: Proposed model for IgM based on the molecular weight data for the papain, peptic, tryptic and chymotryptic fragments and subunits. (Taken from Dorrington and Mihaesco, 1970).

Chapter 2.

The high temperature tryptic fragmentation of normal and monoclonal IgM and the column chromatography purification of their respective Fab μ and Fc $_5\mu$ fragments are described in Chapter 3 and the effect of NaCl concentration and digestion time on the yields of these fragments is outlined. Reduction and alkylation studies on normal and monoclonal IgM and the purification of their respective 7-S subunits, μ -chains and monomeric Fc μ fragments are described in Chapter 4.

Proteolytic fragments and the constituent polypeptide chains of normal and monoclonal IgM, purified as outlined in Chapter 3 and 4, were characterized by ultracentrifugation, amino acid analysis and circular dichroism studies. These results are presented in Chapter 5.

These procedures have resulted in the isolation, in immunogenically pure form, of IgM from Cohn fraction III of pooled normal plasma and in the production of a monospecific anti-IgM antiserum. It was shown by means of various methods of physico-chemical analysis that normal IgM and a monoclonal IgM and their tryptic fragments and μ -chains are comparable in most instances. As far as the author is aware this is the first direct comparison of this kind between normal and monoclonal type IgM molecules and it is rewarding to find that these are similar and that structural and conformational details determined for Waldenström macroglobulins may be extrapolated to normal IgM with some assurance.

Chapter 2

ISOLATION OF NORMAL HUMAN IgM FROM COHN FRACTION III AND OF A MONOCLONAL IgM FROM MACROGLOBULINAEMIC PLASMA

2.1 INTRODUCTION

As indicated in the introductory chapter, the isolation of normal IgM would be of importance for comparative structural and conformational analyses and have possible clinical application. With this in mind preliminary studies on the isolation of normal IgM from Cohn fraction III of pooled plasma was attempted and formed the basis of an earlier report (van der Hoven, 1971). These findings were consolidated and expanded upon in the present investigation and resulted in a reproducible and successful isolation procedure for IgM from Cohn fraction III (van der Hoven et al., 1972). It involves extraction, delipidation, column chromatography and final purification by means of immuno-adsorption. Isolation problems, resulting from the presence of protein complexes, are outlined. The possible nature and origin of these complexes are also discussed.

Monoclonal IgM, in contrast to normal IgM, is obtainable in large amounts from the plasma of patients suffering from Waldenström's macroglobulinaemia, and being homogeneous, is well-suited for detailed chemical and biophysical analysis. The homogeneity and relatively high concentration of these proteins also facilitate their isolation, since the plasma IgM level of macroglobulinaemic patients may rise as high as 7g/100ml (Lawson, 1968). Chemotherapy with hydrocortisone, chlorambucil, cyclophosphamide or phenylalanine mustard can be employed (Solomon and Fahey, 1963), but another method to relieve these patients of various complications connected with a hyperviscosity syndrome, is that of plasmapheresis. Plasmapheresis entails the removal of the

donor's plasma whilst the erythrocytes are reinfused. In a study on the long-term effects of repeated plasmapheresis, Simson et al. (1966), showed that the weekly donation of one litre of plasma had no noticeable effect on the plasma protein levels of healthy human donors. Patients with Waldenström's macroglobulinaemia or multiple myeloma may have an impaired normal antibody response (Fahey et al., 1965), and in order not to aggravate this condition, no more than one litre of plasma per week should be removed in long-term therapy.

In the present investigation, monoclonal IgM was purified from the plasma of a 56-year old man suffering from Waldenström's macroglobulinaemia. At the time of writing this patient had undergone a double plasmapheresis (yielding 1 L plasma) once a week for the past year and complaints of easy fatigue, dizziness, blurred vision and bleeding of mucous membranes have disappeared. The IgM isolated from the plasma of this patient will be referred to as IgM(Sad).

The isolation procedure of IgM(Sad) was similar to that of normal IgM, except that the immunoadsorption step was found unnecessary. Both normal IgM from Cohn fraction III and monoclonal IgM(Sad) were obtained in immunogenically pure form by the procedures outlined in this chapter.

2.2 MATERIALS

Deep-frozen (-30°C) Cohn fraction III of pooled normal plasma was obtained from the Fractionation Unit of the Natal Institute of Immunology.

Plasma containing monoclonal IgM in concentrations varying between 35% and 40% (2.5-2.8g/100ml) of that of the total plasma protein (determined by means of cellulose acetate membrane electrophoresis, Fig. 8), was obtained from the Donor Section of the Natal Blood Trans-

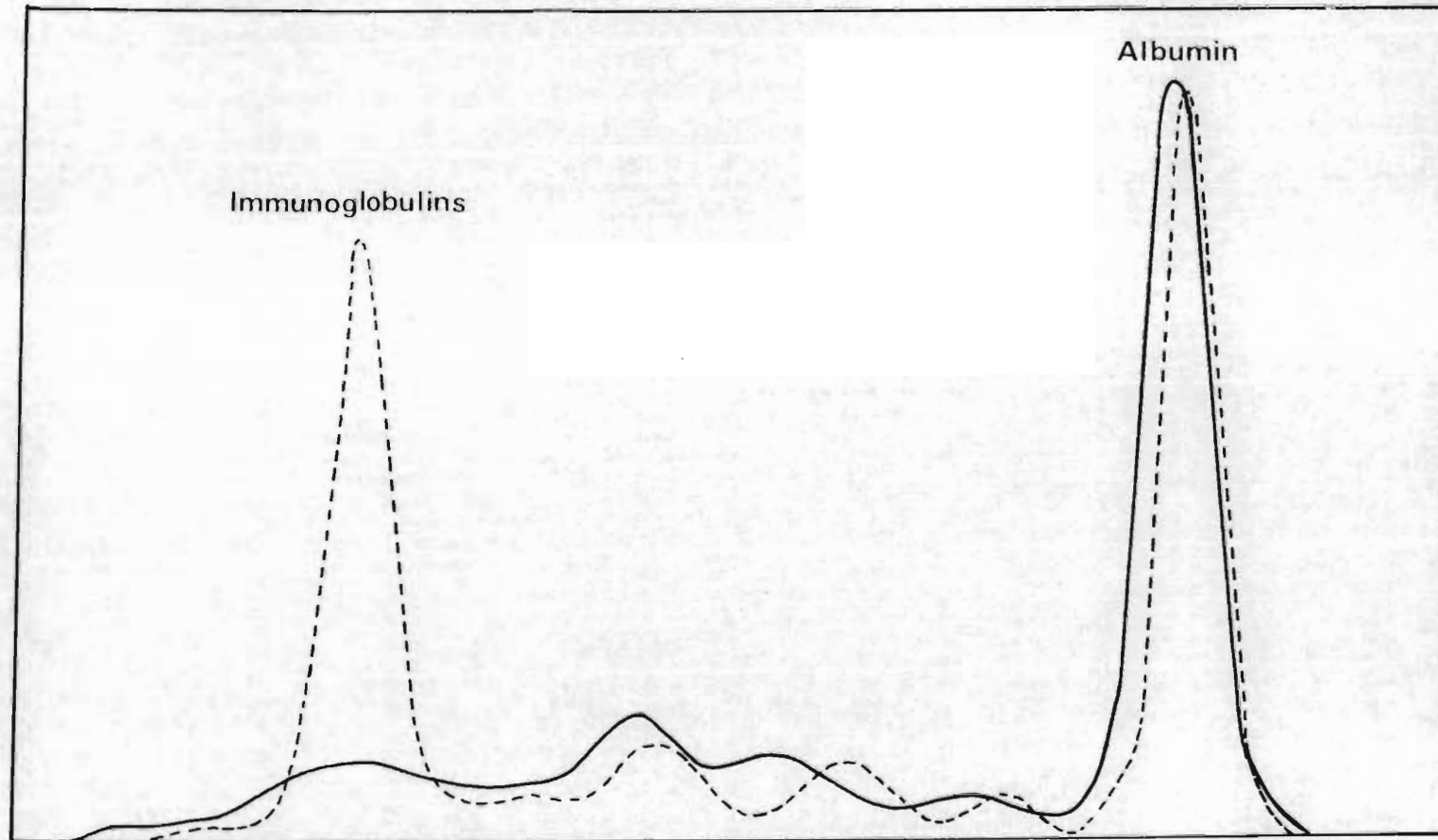


Fig 8: Densitometer tracing of the cellulose acetate membrane electrophoresis of normal serum (—) and Waldenström macroglobulinaemic serum (.....).

fusion Service. This plasma was stored deep-frozen (-30°C).

Commercial antisera monospecific for $\text{Fab}\gamma$, $\text{Fc}\gamma$, IgM , IgA , and IgG (Hylands Laboratories, California, U.S.A.), α_2 -lipoprotein, α_2 -macroglobulin and complement-C3/C4 (Dutch Red Cross Laboratories, Amsterdam) were purchased.

Difco Special Agar Noble (Difco Laboratories, Michigan, U.S.A.) was used for the preparation of agar beads.

Emulphor EL was obtained from Badische Anilin & Soda Fabrik, Germany, and Oxoid I.D. Agar tablets for immunoelectrophoresis from Oxoid Ltd., London.

Sephadex G-100 and G-200 were products of Pharmacia, Uppsala, Sweden, and DEAE (D32) and CM-cellulose (CM32) of Whatman Co., Maidstone, England.

Polyethylene glycol of molecular weight 4,000 and agarose were purchased from Seravac Laboratories, Cape Town.

All reagents were of analytical grade except for toluene, carbon tetrachloride and ethanol which were of reagent grade.

Sodium azide (0.02%) and sodium merthiolate (0.01%) were added as preservatives to all buffers used in molecular exclusion chromatography.

2.3 METHODS

2.3.1 Absorbance measurements and estimation of specific extinction coefficients

The A_{280} of protein solutions was routinely measured in a Beckman DB-G spectrophotometer in 1 cm path length quartz cuvettes.

The specific extinction coefficients, $E_{280}^{1\%}$ of solutions of $\text{IgM}(\text{Sad})$ and normal IgM were determined as follows: Ten ml. of an



IgM(Sad) solution was dialysed against three 2-L volumes of 0.1 M sodium acetate buffer, pH 5.6, for 48 h and the absorbance was determined at 280 nm after filtration through a millipore AA (0.8 μ) membrane filter (Millipore Corp., Massachusetts, U.S.A.). Only the second half of the filtrate was used for absorbance determinations in order to avoid UV - absorbing material possibly released from the filter, influencing the readings. The exact weight of protein in solution was determined as follows: Five 1-ml aliquots of both protein solution and dialysis buffer were lyophilysed to constant weight over P₂O₅ in preweighed weighing bottles. The mean difference in dry weight between protein solution and buffer represents the dry weight of protein contained in 1 ml of solution and was used to calculate the $E_{280\text{nm}}^{1\%}$ value of IgM(Sad). This was found to be 13.1 and is in good agreement with values reported for other Waldenström IgM preparations (Metzger, 1970).

Exactly the same procedure was followed for normal IgM except that a 0.075 M Tris buffer, pH 8.0 was used in this case, and its $E_{280\text{nm}}^{1\%}$ was calculated to be 13.4.

2.3.2 Isolation Procedures

2.3.2.1 Extraction of normal IgM from Cohn fraction III

The method of extraction was the same as that reported by van der Hoven et al. (1972). Frozen Cohn fraction III was ground up in an electric mincer and 500g of this material was extracted for 5h at 4°C with 2L of 0.1M-sodium acetate buffer, pH 4.1, with continuous stirring. The resultant yellow suspension was clarified by centrifugation at 35,500xg (Spinco model L3-50 preparative ultracentrifuge, Rotor 15) for 1h at 4°C. This yielded a large yellow pellet and a slightly turbid,

greenish supernatant topped by a layer of lipid material. The relative proportions of these three phases varied from batch to batch of Cohn fraction III. The supernatant was collected by careful decantation and any lipid particles were siphoned off with a 12-gauge syringe needle connected to an aspirator pump.

2.3.2.2 Euglobulin precipitation of normal IgM

The clarified extract obtained from 500g of Cohn fraction III (1.5 to 1.8L) was dialysed for 24h at 4°C against 20 L of 0.05 M-sodium phosphate buffer, pH 6.5, followed by another 24h period of dialysis against 20 L of 0.001 M-sodium phosphate buffer, pH 6.5. The first buffer of higher ionic strength ensured quick equilibration at the desired pH. The euglobulin precipitate was recovered by centrifugation at 20°C (MSE model 6L preparative centrifuge, 2,000xg, 15 min) and was dissolved in 1.5 L of 0.05 M-sodium phosphate-0.1 M-NaCl buffer, pH 7.0, and subjected to another cycle of euglobulin precipitation. The method used was essentially that of Vaerman et al. (1963).

2.3.2.3 Polyethylene glycol precipitation of normal IgM

Polyethylene glycol (molecular weight = 4,000) was dissolved at room temperature in the clarified Cohn fraction III extract in a ratio of 1:10 (w/v) and the fine precipitate obtained was aggregated by addition of solid NaCl to a final concentration of 0.1 M. The precipitate was removed by centrifugation (2,000xg, 15 min), redissolved in 750ml of 0.05 M-sodium phosphate-0.1 M-NaCl buffer, pH 7.0, and was again precipitated by addition of PEG (8%, w/v). The last step was repeated once more. The optimum PEG concentration to be used for precipitation had been determined previously

by testing (Ouchterlony double diffusion) the supernatants obtained after precipitation of macromolecules at various PEG concentrations for the absence of IgM.

2.3.2.4 Polyethylene glycol precipitation of IgM(Sad)

The IgM of macroglobulinaemic plasma obtained from a single plasmapheresis (400 to 500ml) was precipitated at room temperature by slow addition of PEG 4,000 to a final concentration of 7% (w/v). This represents a minor modification of the method of Polson (1964) in that PEG of a higher molecular weight (6,000) was used by him. The precipitate, containing almost pure IgM, was removed by centrifugation (2,000xg). Because of the ready availability of this plasma and its high concentration of IgM, quantitative recovery was not attempted; the aim was rather to obtain material of as high a purity as possible with the initial precipitation step.

2.3.2.5 Delipidation

Sufficient macroglobulin precipitate was dissolved in 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0 to give a solution containing approximately 5g protein/100ml. The solution was brought to a density of 1.2g/ml by addition of solid NaBr to a concentration of 23% (w/v). Lipoproteins were removed by ultracentrifugal flotation at 105,000xg for 15h at 20°C (Spinco rotor 30). Approximately three quarters of the infranatant was collected by bottom puncture of the centrifuge tubes. Cohn fraction III extract delipidated in this manner always yielded a tightly packed "skin" of lipoproteins at the surface of the centrifuged solution,

whereas a small pellet of greenish material was sometimes observed. The solution in the bottom quarter of the centrifuge tubes was usually very viscous. The lipoprotein layer observed when monoclonal IgM was delipidated was generally quite small.

2.3.2.6 Molecular exclusion chromatography

Agar and agarose beads (3.5%, w/v) were prepared according to the method of Hjertén (1964). By using a 20-L glass aspirator bottle, the preparation of 6 to 7 L of beads at a time was feasible. Principally this method involved the vigorous mixing of an aqueous agar solution with a mixture of organic solvents (in this case carbon tetrachloride and toluene) in the presence of an emulsifying agent (Emulphor EL) at about 50°C. Once agar droplets of sufficiently small size were formed, the temperature of the mixture was lowered as rapidly as possible until gelling occurred at about 32°C. The size of the beads can be controlled by the relative proportions of organic solvents and emulsifier as well as by the speed of stirring. Beads were collected by centrifugation, washed with 95% ethanol and then in water. The beads were finally equilibrated with the eluting buffer, 0.05 M-Tris-0.5 M-NaCl, pH 8.0.

A 10 x 100 cm glass column (Pharmacia, Uppsala, Sweden) with a capacity of approximately 7 L, was packed with the aid of an extension tube with 3.5% agar beads equilibrated with 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0.

Delipidated macroglobulin solution obtained from Cohn fraction III and containing approximately 8 to 10g protein

in a volume of 200-250ml buffer ($E_{280\text{nm}}^{1\%} = 13.4$ used for this calculation) was applied to the column without prior removal of NaBr and was eluted by downward flow with the equilibrating buffer (room temperature; flow rate 200ml/h). Fractions of 25ml were collected and the absorption at 280nm was determined for every fifth fraction on a Beckman DB-G spectrophotometer.

The white gel-like precipitate of IgM(Sad) obtained by PEG precipitation was weighed and dissolved in sufficient eluting buffer to give an approximately 5% (w/v) protein solution. This precipitate contained approximately 20% protein by dry weight as calculated from a value of $E_{280\text{nm}}^{1\%} = 13.1$ which was determined for IgM(Sad). Two hundred and fifty millilitre portions of this solution (12.5g protein) were routinely chromatographed.

2.3.2.7 Concentration of protein solutions

Protein solutions were concentrated by preevaporation in dialysis bags at room temperature, followed by further concentration, if necessary, on PM-30 membranes in an Amicon Diaflo ultrafiltration apparatus (Amicon Corp., Massachusetts, U.S.A.).

2.3.2.8 Immunoabsorption

Crude, immunoglobulin-rich fractions ($1.9 \text{ M}-(\text{NH}_4)_2\text{SO}_4$ precipitates) of goat or rabbit antisera, human IgG prepared by DEAE-cellulose chromatography (Peterson and Sober, 1962) or purified normal IgM were coupled to 3.5% agarose beads according to the cyanogen bromide method of Cuatrecasas (1970). This method of insolubilising biologically active proteins was found to be superior to several other methods tested by Boegman and Crumpton (1970) and coupling efficiencies in

excess of 95% were routinely achieved in the present investigation.

Agarose beads were treated with BrCN in order to activate them for the subsequent coupling with immunoglobulin. Activation depends upon the formation of unstable reactive imino-carbonic acid esters from two vicinal hydroxyl groups of the carbohydrate moiety of agarose and BrCN (Axen et al., 1967). The reaction was carried out at pH 11 and was usually complete after 10 to 12 minutes. Because of the lability of the imino-carbonic acid esters, excess BrCN was quickly washed from the activated agarose beads with cold (4°C) 0.1 M- NaHCO_3 buffer, pH 9.0, and the protein to be insolubilised was then immediately added either in powder form or as a concentrated solution. After stirring the reaction mixture for 20h at 4°C , uncoupled protein was removed by washing with cold (4°C) 1N-propionic acid until the absorbance (280nm) was less than 0.02. The immunoabsorbent was then equilibrated with 0.5M-Tris-0.5M-NaCl buffer, pH 8.0, until no more protein was eluted.

After use, immunoabsorbents were regenerated by washing with propionic acid and buffer, as described above. Desorbed proteins of interest were recovered by titrating the first two propionic acid washes to pH 7.0 with 1N-NaOH and freeze-drying after dialysis against distilled water. Immunoabsorption was carried out in a batchwise procedure at room temperature and was repeated until the specific antibody or antigen to be removed was completely adsorbed. This was tested for after each immunoabsorption, by double diffusion analysis against the appropriate antigen solution or antiserum. These

immunoabsorbents were re-equilibrated and could be used repeatedly (at least 10 times) since they were found to retain their activity for periods of up to a year.

The only antibody neutralization step in which insolubilised antigen was not used, was the first one outlined in Fig.11. This was done as follows: Increasing weights of lyophilised L-chain was added to 1 ml aliquots of antiserum and after incubation at 37°C for 0.5h was allowed to stand at 4°C overnight. The supernatant obtained after centrifugation of the antibody-antigen precipitates was assayed for residual anti-L-chain activity by double diffusion in agar against a 1% (w/v) solution of L-chain. Lack of a precipitin line indicated that no anti-L-chain specific antibodies remained in the supernatant antiserum and a 10% excess of L-chain powder was then considered sufficient for complete neutralization of these antibodies. The precipitin lines found in these experiments were diffuse and generally of poor quality. A ratio of 3mg L-chain powder: 1 ml anti-milk antiserum was arrived at in this manner.

2.3.3 Preparation of sub-fragments of IgG

Light-chain and the Fc and Fab-fragments of pooled normal IgG (Cohn fraction II, Natal Institute of Immunology) were prepared according to the methods of Fleischman et al. (1963) and Porter (1959). In order to obtain L-chain of high purity, free from H-chain, this fraction was subjected to a second cycle of chromatography (Sephadex G-100 superfine) and only the trailing half of the L-chain peak was used. The Fc γ and Fab γ fragments were also rechromatographed (DEAE and CM-cellulose ion-exchangers) until

their purity was established in double diffusion experiments against monospecific anti-Fab γ and anti-Fc γ antisera.

2.3.4 Antiserum Production

2.3.4.1 General immunization procedure

Antisera against the Fab and Fc-fragments of IgG, immunogenically pure IgM and the non-IgM proteins in pool A (see Results) were prepared as follows:

Rabbits were immunized by injecting 0.1mg of antigen in Freund's complete adjuvant.. One quarter of the dose was injected intramuscularly into each hind leg and $\frac{1}{8}$ of the dose at each of four sites in the back muscles, two sites on either side of the spinal column. A booster dose of 10mg, injected in a similar manner, was given one month later. Animals producing satisfactory antisera were plasmapheresed twice per week.

Goats were immunized in the same manner, except that a booster dose of 40-50mg was used.

2.3.4.2 Production of anti-milk antisera

Human milk from 30 donors was pooled, treated at 67°C for 30 min and lyophilysed. A 1.5g sample was dissolved in 12ml distilled water and centrifuged at 4°C for 1h at 3,000xg (Wifug, bench centrifuge). The surface lipid layer was discarded and 1 ml of the infranatant fluid was injected intramuscularly into goats at six sites along the spinal column after homogenisation with an equal volume of Freund's complete adjuvant. The injections were repeated until a satisfactory antiserum was produced. A

period of one week was allowed between injections. Antisera were collected weekly by plasmapheresis.

2.3.5 Immunodiffusion and immunoelectrophoresis

Agar was dissolved (1%, w/v) in 0.05 M-Veronal buffer pH 8.6 for the preparation of slides for immunoelectrophoretic and immunodiffusion analyses. Immunoelectrophoresis (IE) was carried out according to the method of Scheidegger (1955) for 1.5h at 3 mA/slide and 200 volts. The electrode buffer was the same as that used for preparing the agar slides.

Double diffusion in agar was done according to Ouchterlony (1960). Protein solutions for immunological analysis were generally made up as 1% (w/v) solutions in 0.05 M-phosphate-0.1 M-NaCl buffer, pH 7.0.

Photographs of the precipitin lines on the slides were made against a dark background with indirect lighting. A Polaroid-Land MP-3 camera and either Polaroid type P/N 55 or 107 film were employed for this purpose. Best results were obtained if the agar slides were well rinsed and then submerged in water in a specially-constructed shallow glass tank during photography, this having the effect of eliminating edge reflections (compare, for example, Plates 1 and 4).

2.4 RESULTS

2.4.1 Precipitation of normal IgM

Euglobulin precipitation of Cohn fraction III extract has the merit of being a mild procedure, but suffers from the disadvantage that not all IgM species are precipitated under these conditions and is a rather time-consuming procedure. Although this technique

was used extensively during the early stages of this investigation (van der Hoven, 1971), it was later discontinued in favour of precipitation by PEG with its greater ease of operation and higher yields of IgM. Furthermore, solutions of PEG precipitates were less turbid than those obtained from euglobulin precipitates. Turbidity was interpreted as a sign of protein aggregation and caused difficulties during subsequent chromatography experiments and absorbance measurements.

2.4.2 Column chromatography of normal IgM

Molecular exclusion chromatography of either the PEG or euglobulin precipitate on agar beads yielded the typical elution profile shown in Fig. 9 which corresponds with the earlier findings of van der Hoven (1971) using euglobulin precipitates. The first turbid peak consisted of amorphous particles of aggregated protein and was always considerably bigger for euglobulin than PEG precipitates. The second peak (Pool A, Fig. 9) contained all of the IgM, other high molecular weight species (α_2 -macroglobulin, α_2 -lipoprotein) and, unexpectedly, some lower molecular weight proteins (IgG, IgA, complement). The third peak constituted lower molecular weight proteins like IgA, IgG, complement, albumin and others which were not identified.

The identity of the proteins in Pool A was established as follows:

Pool A was cut into fractions of equivalent volume (Fig. 9, fractions a to f) and concentrated to approximately the same protein concentration. These fractions were examined by means of double diffusion in agar against antisera monospecific for the following antigens: IgM, IgA, IgG, α_2 -macroglobulin, α_2 -lipoprotein and complement (Plate 1a and lower portion of Fig.9). Rechromatography of

$A_{1\text{cm}}^{280\text{m}}$

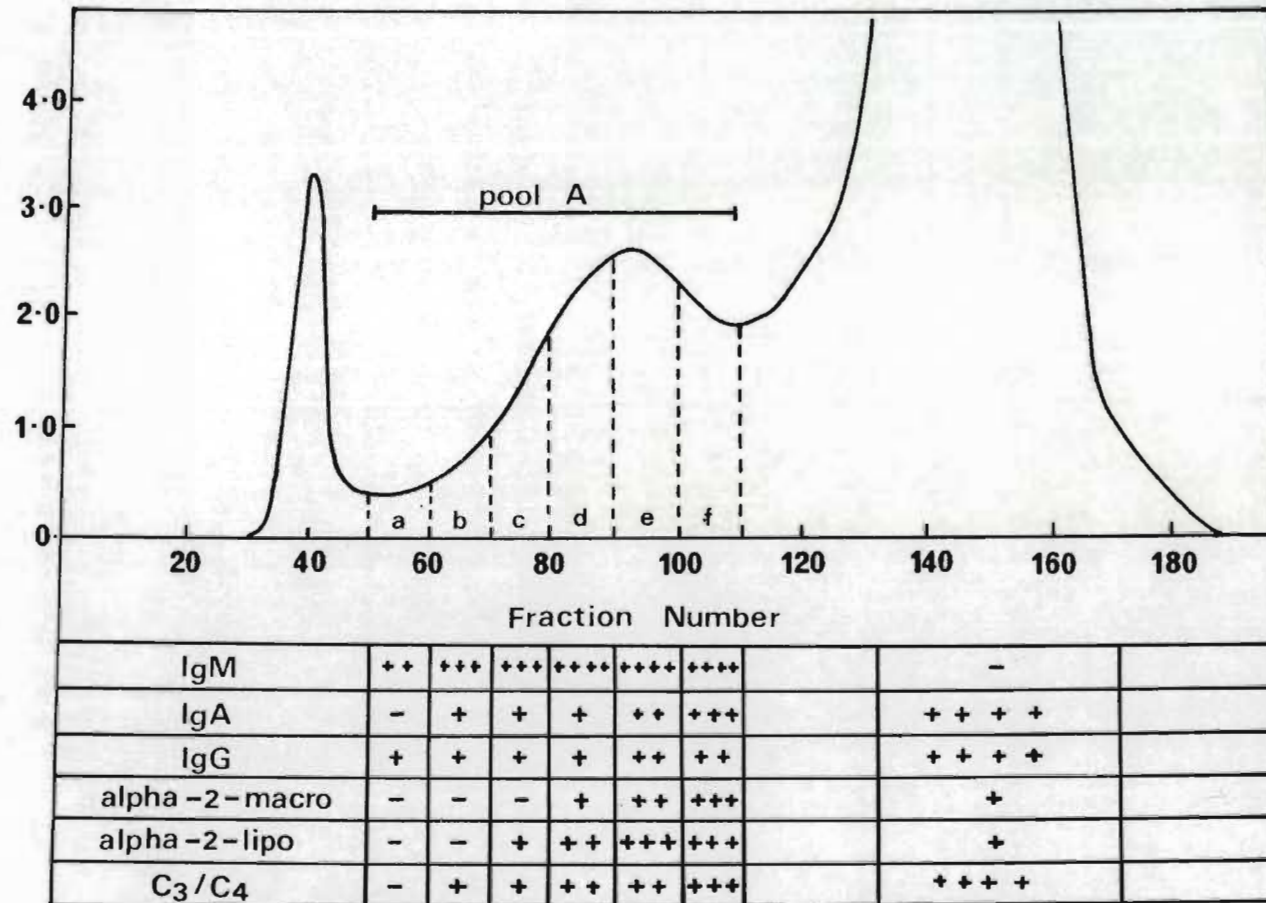


Fig 9: Elution profile obtained after the chromatography of 200 ml of redissolved macroglobulin precipitate from Cohn fraction III extract (approximately 8g protein) on 3.5% agar beads (10 x 100 cm column). Flowrate was 250 ml/h and 25 ml fractions were collected. The relative concentration of IgM, IgA, IgG, α_2 -macroglobulin, α_2 -lipoprotein and complement (C3/C4) of fractions a-f as judged by Ouchterlony analyses against monospecific antisera is shown below each fraction.

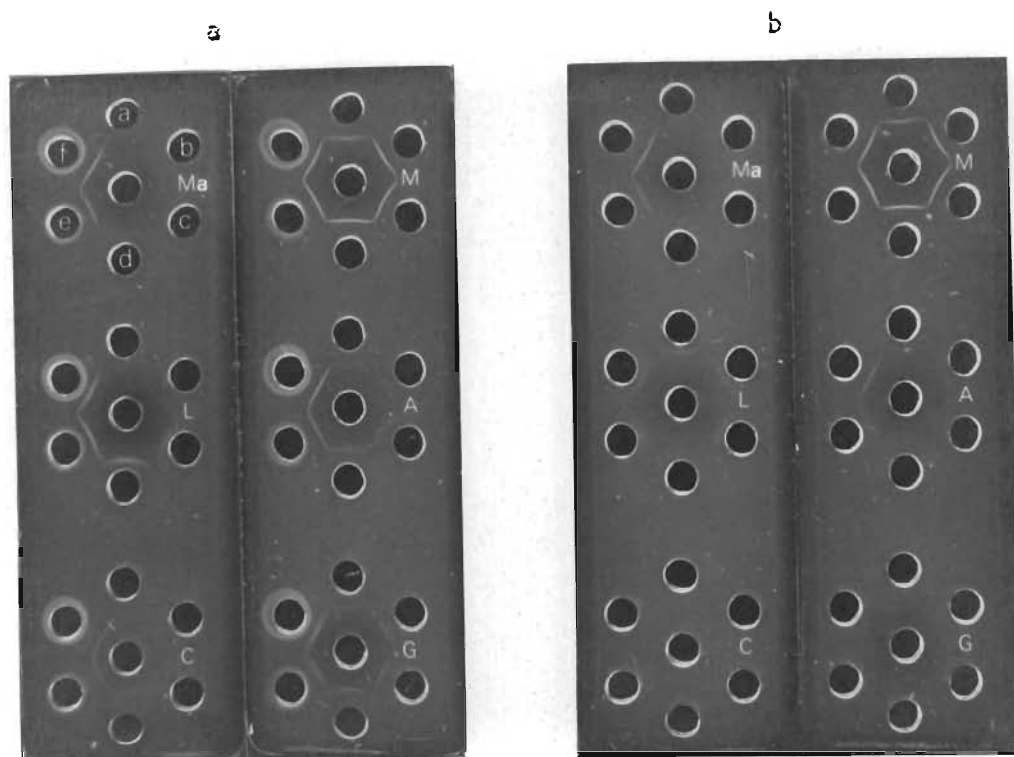


Plate 1: Ouchterlony double diffusion analysis on fractions collected at equivalent elution volumes (Fig. 10) for rechromatographed unheated pool A (a), and pool A heated at 56°C for 1.5 - 2h. (b). Specific antisera to IgM (M), IgA (A), IgG (G), α_2 -macroglobulin (Ma), α_2 -lipoprotein (L) and complement -C3/C4 (C) were placed in the central wells and subfractions a-f as indicated in the top left hand corner.

pool A after concentration gave no enhanced resolution of IgM from the other contaminating proteins as judged by Ouchterlony analyses.

The effect of heat on the chromatographic elution profile of pool A proteins is shown in Fig. 10. The chromatogram clearly shows that heating caused an increase in the amount of material in the turbid peak as well as in the high molecular weight region immediately following it. Immunodiffusion analysis of the equivalent subfractions (Fig. 10) collected from pool A before and after heating (pool A') revealed that heat treatment had decreased the content of α_2 -lipoprotein (this macroglobulin sample was not delipidated), IgA, and IgG in pool A' (Plate 1). Fractions a - c of pool A' shown in Plate 1 appeared to contain only IgM, but rabbit antisera prepared against these fractions invariably also contained anti- γ and anti- α -chain antibodies.

2.4.3 Purification of normal IgM by immunoabsorption

A flowsheet depicting the development of the different antisera and immunoabsorbents is presented in Fig. 11.

Immunoabsorbents made from H-chain specific goat antisera against human milk (immunoabsorbent A - Fig. 11) was used in batch-wise fashion (3 to 4 times) on pool A' and removed all contaminants, yielding highly purified IgM (Plate 2a). The immunoelectrophoretic purity of this IgM preparation is demonstrated in Plate 2b. Yields of 0.5g to 1g immunogenically pure IgM/500g Cohn fraction III were obtained.

Rabbits or goats immunised with this IgM preparation gave a strong anti-immunoglobulin antiserum (Plate 3a) which could be made monospecific for μ -chain (Plate 3b) by adsorption with insolubilised IgG (γ +L-chain, Immunoabsorbent B - Fig. 11). These results have been previously reported (van der Hoven, 1971).

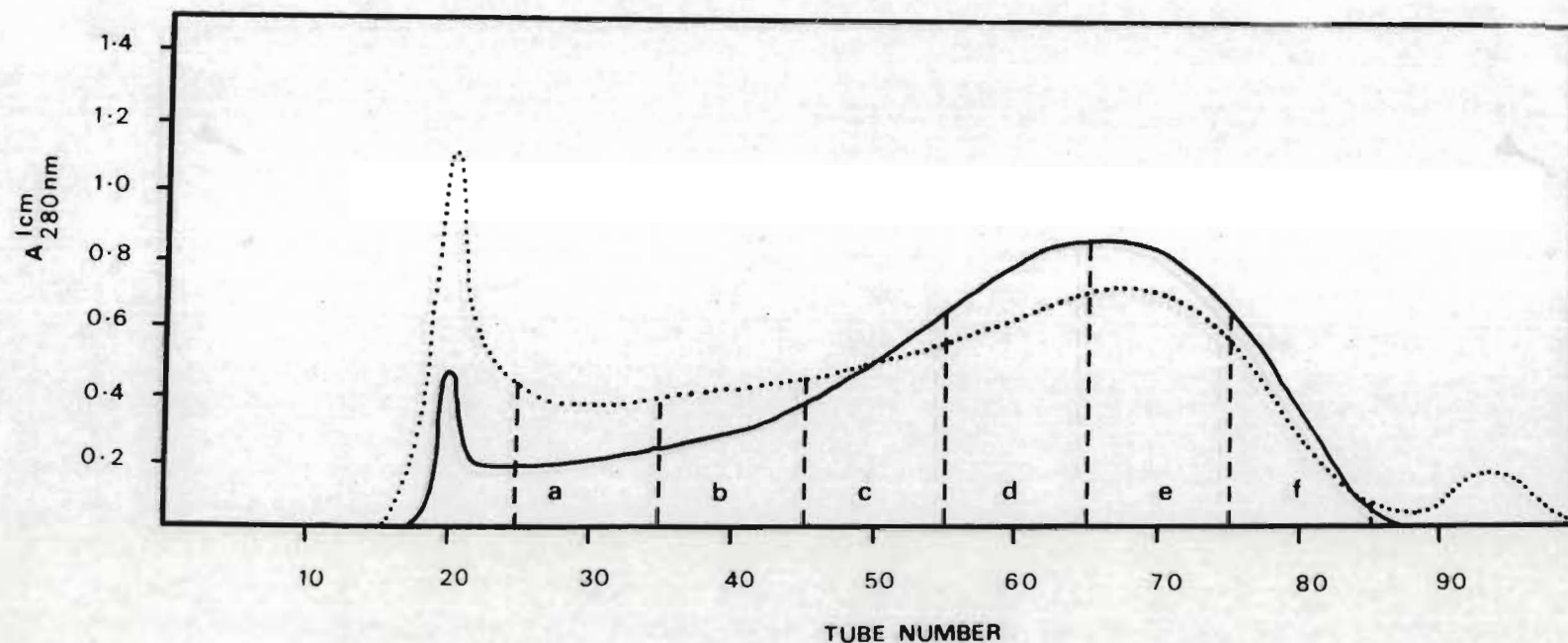


Fig. 10: Elution profiles obtained for rechromatographed pool A (3.5% agar beads; 5 x 100 cm column).

Unheated pool A: (—).

Pool A heated at 56°C for 1.5 - 2 h: (.....).

Flowrate was 100 ml/h and 10 ml fractions were collected.

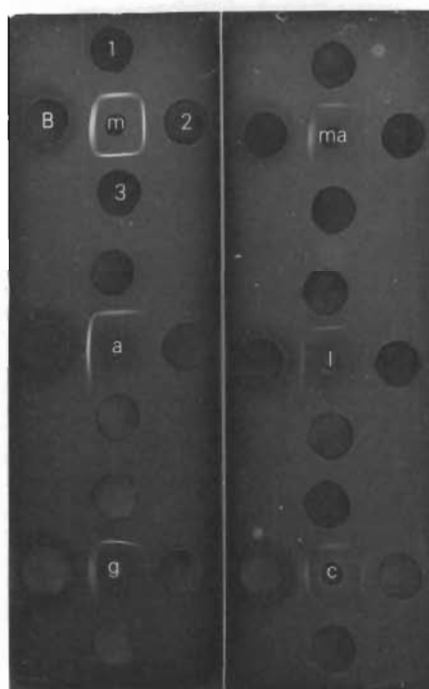


Plate 2a: Ouchterlony double diffusion analysis of pool A' proteins before (B) and after the first (1), second (2) and third (3) immunoabsorptions with insolubilised, H-chain specific, anti-milk antiserum (peripheral wells), against antisera monospecific for IgM (m), IgA (a), IgG (g), α_2 -macroglobulin (ma), α_2 -lipoprotein (l) and complement (c).



Plate 2b: Immunoelectrophoretic patterns obtained for normal serum (NS) and IgM purified from pool A (A), against anti-total serum.



Plate 3: Immunoelectrophoretic patterns obtained for normal serum (NS) and pool A (A) against anti-IgM as prepared in Methods.

- (a) Unadsorbed anti-IgM
- (b) Anti-IgM adsorbed with insolubilised IgG (γ +L-chain)

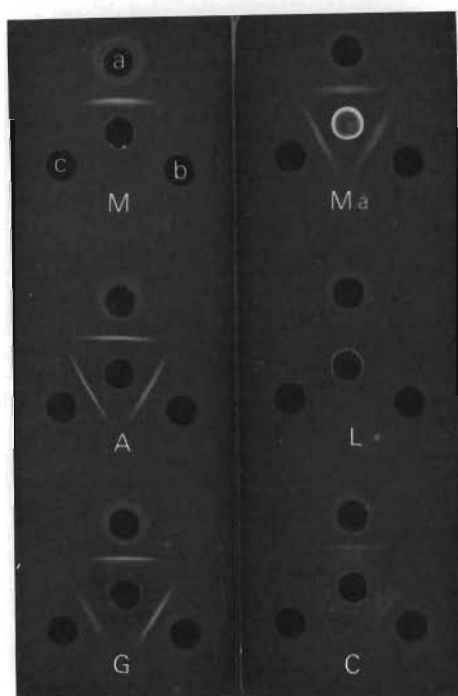


Plate 4: Ouchterlony double diffusion analysis of:

- (a) pool A;
- (b) pool A after first immunoabsorption with insolubilised anti-IgM;
- (c) pool A after second immunoabsorption with insolubilised anti-IgM;

Specific antisera to IgM (M), IgA (A), IgG (G), α_2 -macroglobulin (Ma), α_2 -lipoprotein (L) and complement (C) were placed in the centre wells as indicated.

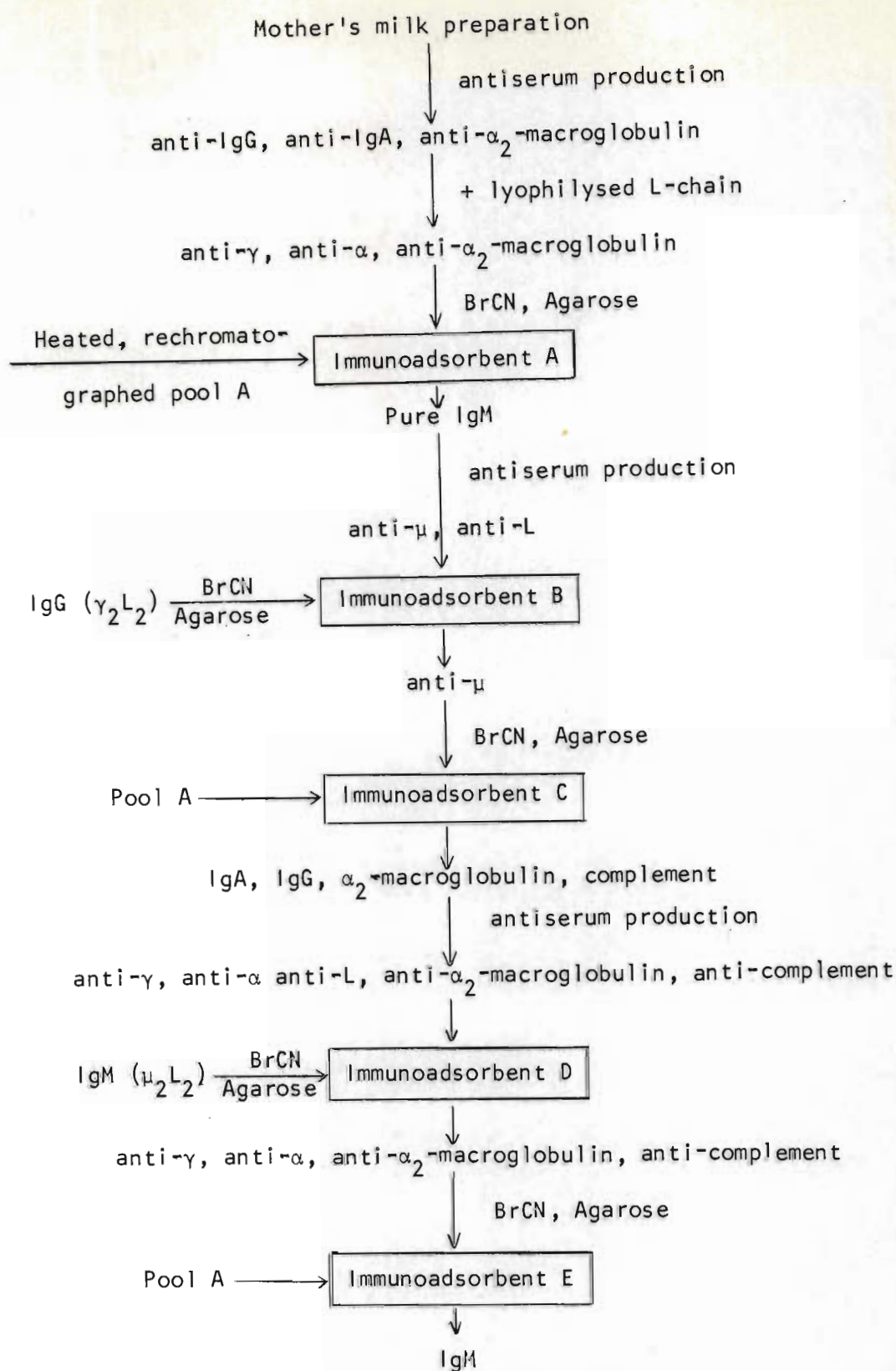


Fig. 11 Flowchart of the sequential development of antisera used in the isolation of immunogenically pure IgM from Cohn fraction III of pooled normal human plasma. The Greek letters γ , α and μ indicate the heavy chains of IgG, IgA and IgM respectively and L the light chains (κ and λ) of immunoglobulins.

Once monospecific anti-IgM (i.e. anti- μ -chain) antiserum was prepared, a more refined and specific immunoabsorbent was made against the non-IgM proteins in pool A. Insolubilised anti- μ -chain antiserum (Immunoabsorbent C - Fig. 11) was used to render pool A completely deficient in IgM (Plate 4). The residual proteins (i.e. pool A "contaminants") were next used as antigens for antiserum production (Plate 5a).

Immunoabsorbent prepared from this "second generation" antiserum (Immunoabsorbent E - Fig. 11) contained antibodies against contaminating proteins only, after being made H-chain specific. In this case anti-L-chain antibodies were neutralised (Plate 5b) by immunoabsorption with insolubilised IgM (μ +L-chain, Immunoabsorbent D - Fig. 11). This step not only removed anti-L-chain antibody, but also ensured the removal of any possible anti- μ -chain antibody that may still have been present. With immunoabsorbent E, up to 2g immunogenically pure IgM could be isolated from 500g Cohn fraction III. This represents a recovery of 24% of the available IgM in Cohn fraction III and is based on an average concentration of 530mg IgM/100 ml of Cohn fraction III extract as prepared for euglobulin or PEG precipitation. It must be stressed however, that this concentration was obtained by means of the single radial immunodiffusion technique of Mancini et al. (1965), and that the yield figure represents an optimum value in view of the complexity and variability of the proteins extracted from Cohn fraction III.

Proteins desorbed from immunoabsorbent E were examined by means of immunoelectrophoresis and double diffusion in agar. The expected pool A "contaminants" could readily be identified by double diffusion analysis, but the presence of IgM was rather



Plate 5: Immunoelectrophoresis of normal serum proteins versus antiserum against non-IgG1 contaminants (see Plate 4) in pool A:

- (a) unadsorbed antiserum;
- (b) antiserum adsorbed with insolubilised IgM.

surprising (Plate 6). A comparison of the precipitin arcs yielded by this material and normal serum against antisera to IgA, IgG, IgM and α_2 -macroglobulin (Plate 7), showed distinct differences in the mobilities of some of these antigens and in the relative positions of their precipitin lines. These differences were best illustrated by the IgG (Plate 7b) and α_2 -macroglobulin (Plate 7d) precipitin lines and could be considered as indications of complex formation between the various macromolecules in pool A (see Discussion).

2.4.4 Column chromatography of IgM(Sad)

A typical chromatogram obtained from an agar column for the PEG precipitate of IgM(Sad) is shown in Fig. 12. After conservative pooling of only those fractions in the shaded area of the peak (Fig. 12), about 50% of the applied protein was recovered in highly purified form. This is shown by the immunoelectrophoretic analysis of this material against anti-total serum, which gave only a single precipitin arc characteristic of IgM (Plate 8).

2.5 DISCUSSION

The high molecular weight of IgM in comparison with that of most other plasma proteins suggests that it could be easily separated from them. Only proteins like α_2 -macroglobulin, α_2 -lipoprotein, β -lipoprotein (Schultze and Heremans, 1966), polymeric IgA (Vaerman et al., 1965) and possibly the C1 component of complement (Naff et al., 1963) have molecular weights in the same range as that of IgM. Gradient ultracentrifugation has therefore been used for the isolation of IgM from animal (Parkhouse et al., 1970) and human serum (Vaerman et al., 1963). Not only does this technique separate the 18-S proteins from

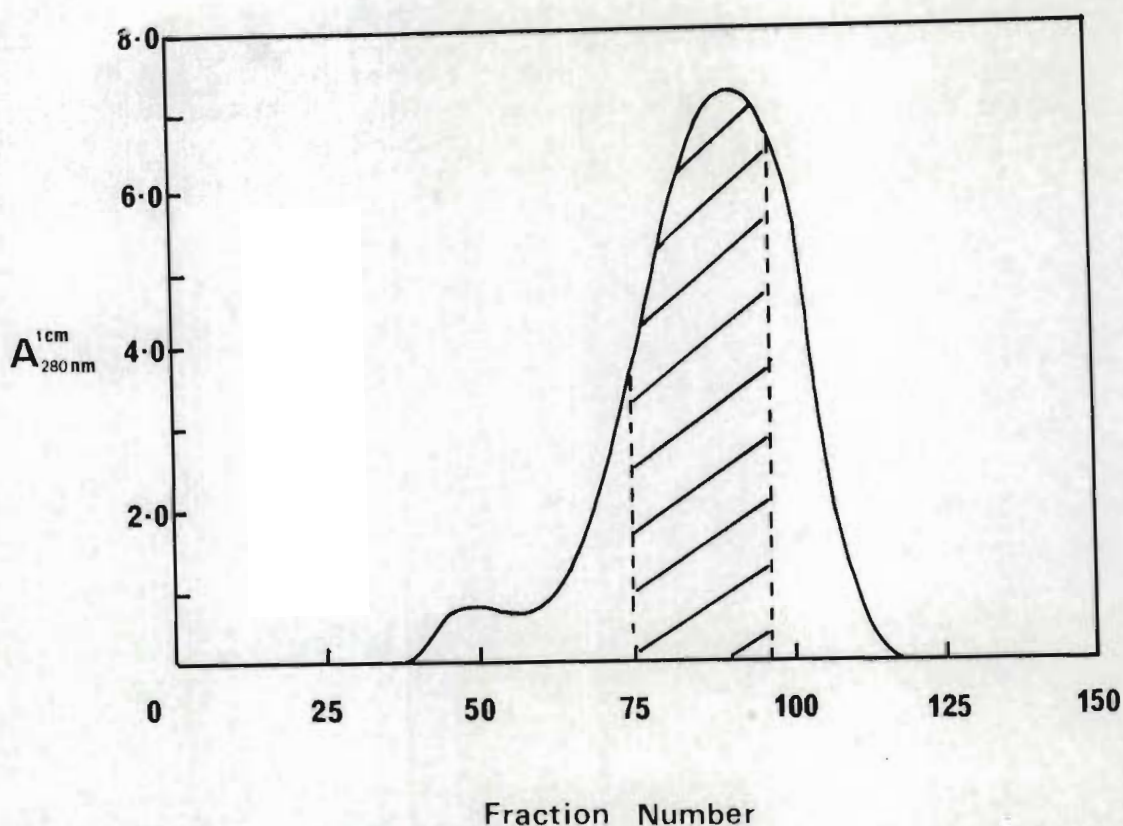


Fig. 12: Elution profile obtained after chromatography of 250 ml of redissolved PEG precipitate of Waldenström plasma (approximately 12.5g) on 3.5% agar beads (10 x 100 cm column). Flowspeed was 250 ml/h and 25 ml fractions were collected.



Plate 6: Ouchterlony double diffusion analysis of proteins desorbed from insolubilised antiserum against non-IgM contaminants of pool A (centre well) and monospecific antisera to IgG (G), IgM (M), IgA (A) and α_2 -macroglobulin (Ma).

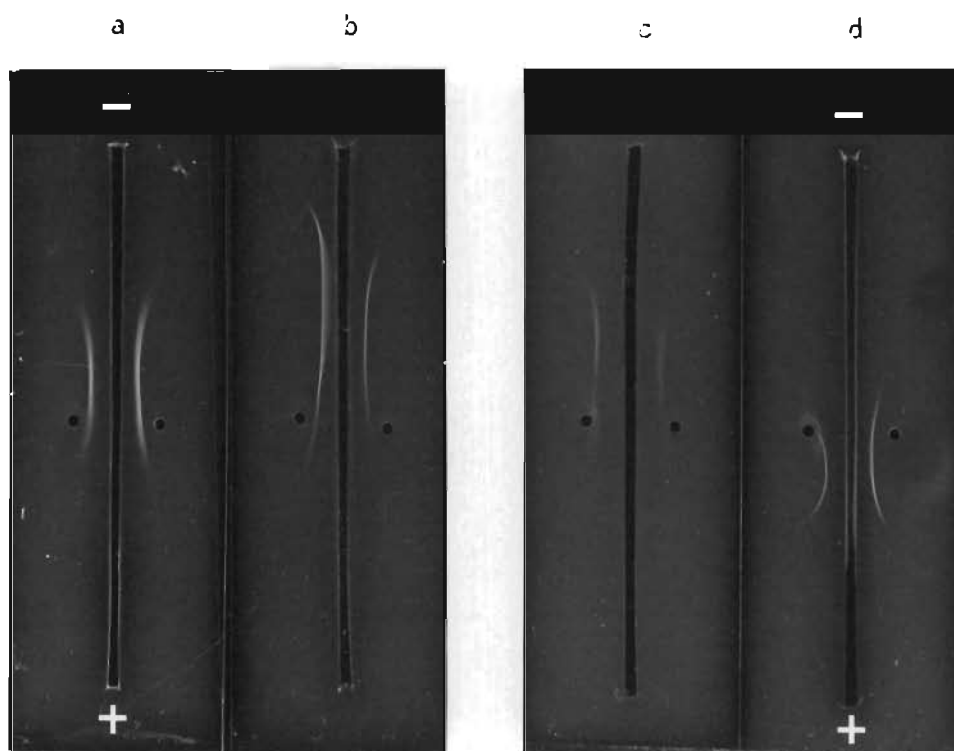


Plate 7: Immunoelectrophoretic patterns obtained for normal serum proteins (left hand wells) and proteins desorbed from insolubilised antiserum to non-IgM contaminants of pool A (right hand wells) against monospecific antisera to IgA (a), IgG (b), IgM (c) and α_2 -macroglobulin (d).



Plate 8: Immunoelectrophoretic patterns obtained for purified IgM(Sad) against anti-total serum.

the lipoproteins, but by careful collection of fractions, IgM was also separated from α_2 -macroglobulin (Vaerman et al., 1963).

Application of this approach unexpectedly yielded IgM preparations heavily contaminated with IgA, IgG, α_2 -macroglobulin and complement when Cohn fraction III was the source material. Ion exchange chromatography (Chaplin et al., 1965) and zone electrophoresis (Müller-Eberhard, 1960; Chesebro et al., 1968) were also attempted, with no better results.

Molecular exclusion chromatography for the purification of IgM has been widely used since Killander et al. (1964), reported favourably on the results of using agar beads for the fractionation of human plasma macroglobulins. The physico-chemical studies reported by Miller and Metzger (1965a) on a Waldenström macroglobulin purified by this means, underlined the practicability of this approach. In the present investigation, preference was therefore given to an agar gel matrix rather than the more popular Sephadex and Biogel preparations on account of agar's higher molecular exclusion limits coupled with good flow characteristics and ease of preparation in large amounts in the laboratory itself.

Although the typical elution profile in Fig. 9 for normal IgM extracted from Cohn fraction III is similar to that found by Killander et al. (1964) upon chromatography of whole serum, the second peak (Pool A, Fig. 9) which contains most of the IgM, was still heavily contaminated by the proteins IgA, IgG and complement (C3/C4). Re-chromatography of pooled material from the middle of the peak (fractions d and e, Fig. 9) on either 3.5% agar or Sephadex G-200 analytical columns did not separate IgM from the contaminants, although their molecular weights are widely different (e.g. 1.5×10^5 for IgG as against 1×10^6 for IgM). These findings together with those obtained

from sucrose gradient ultracentrifugation and zone electrophoresis suggested that the contaminants were present, in part at least, as complexes or aggregates in Cohn fraction III and that this gave rise to their high apparent molecular weights.

Chromatography in the presence of dissociating agents like 8 M-urea was therefore attempted, but this was not successful, because it led to the virtual complete loss of IgM antigenicity, probably because of irreversible changes in conformation. Evidence for the latter explanation was obtained in circular dichroism (CD) studies on purified normal and monoclonal IgM. The CD curves of GuHCl-denatured IgM preparations did not return to those of the native proteins after removal of the denaturant, but resembled those of randomly coiled proteins (cf. Chapter 5).

The known heat lability of α_2 -macroglobulin (Schultze and Heremans, 1966) led to the introduction of a heat treatment step before rechromatography. The improved quality of the pool A' fraction thus obtained (viz. a decreased content of α_2 -lipoprotein, IgA, IgG and complement, Plate 1) eventually made possible the first successful isolation of immunogenically pure IgM from Cohn fraction III (van der Hoven, 1971). It is of course possible that the contaminating proteins might only have lost their native antigenicity upon heating and were in fact still present in pool A', albeit in altered (denatured) form. The observation that only a small extra peak of lower molecular weight proteins eluted on rechromatography of heated pool A' (Fig. 10) provides circumstantial evidence for this possibility. The original motivation for heat treatment of pool A' proteins, viz. removal of α_2 -macroglobulin, was not achieved by this procedure and last traces of this contaminant were eventually removed by immunoadsorption.

Affinity chromatography employing polysaccharide beads as carriers (Axen et al., 1967), is a powerful and mild technique for the purification of proteins on the basis of biological specificities. In the case of the immunoglobulins it has been shown by Mannik and Stage (1971), for instance, that IgG, IgA and IgM may be removed individually or in toto from human serum by this method.

The dominating immunoglobulin in human secretions, including milk, is IgA (Tomasi et al., 1965). In human colostrum, however, IgM is found in almost equal abundance and its concentration probably approaches that of IgA. Furthermore, human milk also contains IgG and C3 (Schultze and Heremans, 1966). During lactation the IgM concentration level drops rapidly until it is almost undetectable (Schultze and Heremans, 1966). Although the anti-milk antiserum was primarily prepared for its anti-IgG, anti-IgA and anti-C3 specificity, it fortunately also contained some anti- α_2 -macroglobulin activity. After rendering this antiserum H-chain specific and insolubilising it, it was an efficient immunoabsorbent with which to purify heated, rechromatographed pool A. Human milk can be considered to be the ideal natural antigen for the production of the antiserum in question. Indeed, the preparation of immunogenically pure IgM (Plate 2b) followed by the production of mono-specific anti-IgM (Plate 3b) was made possible with the aid of such an immunoabsorbent. The anti-IgM antiserum was tested at the International Reference Centre for immunoglobulins of the W.H.O. and was rated excellent (Rowe, 1972).

Notwithstanding the initial success achieved with immunoabsorbent A it was subsequently established that the activity and specificity of later preparations of anti-milk antisera were too variable, especially as far as the removal of α_2 -macroglobulin was concerned. Proteins

desorbed from immunoadsorbent A were therefore used for immunization, but it was found that the antiserum produced could not be used because of its strong anti- μ -chain activity. Efforts to remove the anti- μ -chain antibodies by immunoadsorption with insolubilised IgM(Sad) were unsuccessful. This was not surprising in view of the known restriction in respect of heavy-chain subclass, allotype and idiootype of monoclonal immunoglobulins (Humphrey et al., 1971).

The availability of monospecific goat anti-IgM antiserum, however, cleared the way for preparing a superior antiserum against known as well as undetected contaminants in pool A. The latter antiserum could be made against non-IgM proteins by first rendering pool A completely deficient in IgM with the former antiserum and using the residual proteins as antigens for antiserum production. Moreover, the anti-IgM antiserum also ensured that such an antigen preparation could be reliably produced at any later date.

Although the "second generation" antiserum (Immunoadsorbent D) of necessity contained the specific antibodies to pool A contaminants, it also contained strong anti-L-chain antibodies which would absorb IgM ($= (\mu_2L_2)_5$) as well (Fig. 11). The obvious solution to this problem was to neutralize these antibodies with purified L-chain. However, the difficulty experienced in the preparation of L-chain in large quantities of sufficient purity, i.e. without any γ -chain, led to the investigation of two alternative approaches to get rid of all anti-L-chain specificity in the antiserum:

- (i) Anti-L-chain activity was neutralized with Fab-fragment of IgG. This meant that anti-Fd γ specific antibodies would also be removed, but it was anticipated that the anti-Fc γ antibodies would be unaffected, thereby retaining

the ability to remove IgG from pool A. Pilot experiments, though, showed only very weak anti-Fc γ activity after neutralisation of the antiserum with Faby and, not surprisingly, immunoabsorbent prepared on larger scale was found incapable of removing the IgG component of pool A.

- (ii) Anti-L-chain activity was neutralized by immunoabsorption with insolubilised IgG. This approach rendered the antiserum totally deficient in anti- γ -chain activity, necessitating the production of a second antiserum and immunoabsorbent specifically for IgG extraction. To circumvent the possibility of such an antiserum containing anti-L-chain antibodies, it was made against the Fc-fragment of IgG. It was found however, that anti-Fc γ -immunoabsorbent not only removed an appreciable amount of IgM, but some residual IgG could still be detected afterwards in pool A by means of an anti- γ -chain antiserum. An explanation for these anomalous observations may be that the IgG in pool A existed in the form of at least two different species of soluble complexes:

- (a) IgG may be complexed with IgM. Such a situation could arise if IgG with anti-IgM specificity were to come in contact with its antigen (IgM). Adsorption of IgG complexed in this manner would mean simultaneous removal of bound IgM.
- (b) IgG may be complexed with other proteins in such a way that its Fc-region is entirely masked, making the molecule unrecognisable to the monospecific

anti-Fcy immunoadsorbent; C3 may be implicated in this context (Askenase and Leonard, 1970).

After experiencing these setbacks it was realised that the soundest approach would be to neutralize anti-L-chain antibodies with an IgM immunoadsorbent (Immunoadsorbent D, Fig. 11). Only 0.3g of purified normal IgM was available at the time for this purpose. The stability of immunoadsorbents fortunately provides such a built-in amplification effect that it was eventually possible to prepare 10g of pure IgM employing this single batch as the penultimate immunoadsorbent (D) in the isolation scheme outlined in Fig. 11.

Further evidence of the complexity of the non-IgM contaminants was found when these proteins were recovered and subjected to immunoelectrophoretic analysis against various monospecific antisera. The precipitin arcs that developed against anti- α_2 -macroglobulin and anti-IgG antisera were obviously abnormal when compared with those yielded under the same conditions by normal serum proteins (Plates 7b and 7d).

Although little is known about the nature and composition of the complexes extracted from Cohn fraction III, it is highly likely, for example, that aggregation of IgG can take place during the Cohn fractionation procedure. Treatment with ethanol and other organic solvents is known to cause aggregation and the concomitant appearance of anticomplementary activity in IgG solutions (Waldesbuhl et al., 1970). The anticomplementary activity of Cohn fraction II solution is a well-known phenomenon and various attempts have been made to render this fraction safe for intravenous administration (Barandun et al., 1962; Schultze and Schwick, 1962; Sgouris, 1967). It is generally accepted that the cause of anticomplementary activity lies in the presence of aggregated or semi-denatured monomeric immuno-

globulin molecules and it has been shown that enzyme digestion, reduction and alkylation or separation of aggregated material by sodium sulphate precipitation (Frommhagen and Fudenburg, 1962) yields non-anti-complementary IgG solutions.

Müller-Eberhard and Kunkel (1961), have furthermore shown that heat-aggregated human IgG will bind the first component of complement, C1. Once the complement system has been activated, C3 and C4 can bind to the IgG-C-complex (or to the individual IgG molecule), thereby further increasing the molecular size of the complex (Askenase and Leonard, 1970; Chan and Cebra, 1968; Müller-Eberhard and Lepow, 1965). It is therefore conceivable that a series of reactions involving IgG and some of the complement components may be brought about during the manipulations (including ethanol precipitation) of the Cohn fractionation procedure, and that these could produce soluble complexes with molecular weights in excess of 10^6 daltons. This would explain the unusual presence of IgG, as well as of complement components, in the high molecular weight fraction (Pool A) after agar gel chromatography.

Additional explanations for the presence of complement in pool A could be found in the work of Augener et al. (1971), who have shown that the relative binding capacity of C1 to IgG and IgM is dependent upon the degree of polymerization of these proteins in their native state. Although comparable amounts of C1 are bound by IgG and 7-S IgM, higher molecular weight IgM, such as the species sedimenting at more than 35-S, will bind 116 times more C1. It has also been shown by Borsos and Rapp (1965), and by Cohen (1968), that the minimum unit for complement fixation by IgG consists of two adjacent antibody molecules linked by an antigen bridge whereas a single molecule will suffice in the case of IgM. Complex formation between IgM and complement components is therefore even more likely than for IgG.

IgA, on the other hand, is known not to bind complement (Ishizaka et al., 1966a) but undergoes disulphide interchange reactions with various other serum proteins which give rise to higher molecular weight IgA-containing complexes (Mannik, 1967). Sedimentation coefficients of up to 17-S have been reported for polymeric normal IgA (Vaerman et al., 1965). The unexpected high IgA content of pool A may be explained by these observations.

One additional source of protein complexes in the Cohn fraction III used during this investigation may be that plasma obtained from 400 to 600 individual blood donations (i.e. 100 to 150 L), was usually pooled for fractionation purposes. The probability of naturally occurring antibodies to isogenic plasma proteins meeting their antigens in such a volume of plasma must be considered reasonable, taking into account that the frequency of non-specific antibodies against the gamma globulins alone, has been found to be 5.4% in 1,989 adults tested (Wilson and Steinberg, 1965). The isoantigenicity of the low density lipoproteins, the α_2 -macroglobulins, IgA and C3 (Bütler, 1969) is also well known and may contribute to the formation of soluble complexes in large volumes of pooled plasma.

In contrast to the problems encountered during the isolation of normal IgM, the purification of IgM(Sad) was an easy two-step procedure. This is not surprising in view of the high concentration of this protein in the macroglobulinaemic plasma from which it was isolated. The ease with which monoclonal IgM is generally isolated, is reflected by the fact that many workers utilize only the euglobulin character of these proteins for their isolation (some are however not euglobulins) and do not make use of any additional purification techniques.

The isolation of normal IgM from Cohn fraction III and of a mono-

clonal IgM, IgM(Sad), from the plasma of a patient suffering from Waldenström's macroglobulinaemia had therefore been achieved, employing techniques like euglobulin and PEG precipitation, column chromatography and immunoabsorption. By means of the last-mentioned technique, immunogenically pure normal IgM was isolated in up to 2g quantities from 500g of starting material. Sufficient quantities of highly purified normal and monoclonal IgM were consequently available to undertake the comparative structure and conformation studies described in the following chapters.

Chapter 3

THE DIGESTION OF NORMAL IgM AND IgM(Sad) WITH TRYPSIN AND THE PURIFICATION OF THE TRYPTIC FRAGMENTS

3.1 INTRODUCTION

After the early report of Porter (1959) on the use of papain for the structural determination of rabbit IgG, proteolytic enzymes have come to be widely employed in immunoglobulin research. Digestion by proteases like pepsin, papain, pronase, trypsin, chymotrypsin or thermolysin is today essential for the elucidation of the positions of carbohydrate moieties, the composition in terms of fragments and the primary structure of the immunoglobulins. Application of these enzymes, in conjunction with the necessary techniques for the separation and sequencing of the resultant fragments, has led to the determination of the entire amino acid sequence of IgG (Edelman et al., 1969) and of more than 90% of that of a Waldenström IgM (Putnam et al., 1972).

Human IgG is only digested to a limited extent by papain at 37°C, yielding well-defined Fab γ and Fc γ fragments (Porter, 1967), but IgM is rapidly degraded to a heterogeneous mixture of fragments and peptides by this enzyme (Putnam, 1967), unless very carefully controlled conditions are maintained. Despite the efforts of Onoue et al. (1968) and Mihaesco and Seligmann (1968), yields of Fc μ -fragment in particular were insufficient for detailed physico-chemical and biochemical studies. Such studies became possible only recently after it was discovered by Plaut and Tomasi (1970), that trypsin digestion of IgM at an elevated temperature yields satisfactory amounts of both major fragments (Fab μ and Fc $_{5\mu}$).

The work reported on in this chapter deals with an investigation of

different conditions for the high temperature tryptic fragmentation of normal IgM and IgM(Sad), as well as column chromatographic procedures for the separation and purification of such fragments. The heterogeneity of some tryptic fragments is emphasized, and its possible causes are discussed.

3.2 MATERIALS

Normal IgM and IgM(Sad) were purified as described in chapter 2.

Commercial antisera monospecific for κ and λ light-chains were obtained from Hylands Laboratories, California, U.S.A.

Grade 1 guanidine hydrochloride (GuHCl), sodium dodecyl sulphate (SDS), phenylmethylsulphonylfluoride (PMSF), N-benzoyl-L-arginine ethylester (BAEE) (Sigma Chemical Co., Missouri, U.S.A.), acrylamide, N,N'-methylene diacrylamide (BIS), N,N,N',N',-tetra methylethylenediamine (TEMED) (Koch-Light Laboratories, Buckinghamshire, England) and Pyronin G (Merck Darmstadt, Germany) were purchased from the respective suppliers. All chemical reagents were of analytical grade and were used without further purification, except that SDS and acrylamide were recrystallised from 5% (w/v) solutions in 95% ethanol and chloroform, respectively.

Sephadex G-200 and Sepharose 6B (6% agarose beads) were obtained from Pharmacia, Uppsala, Sweden.

Three times crystallised Bovine pancreatic trypsin (grade 5) was a product of Miles-Seravac Laboratories, Cape Town.

3.3 METHODS

3.3.1 Trypsin assay method

The activity of trypsin in the presence of various concentrations of NaCl at room temperature and at 56°C, was assayed according to the method of Schwert and Takenaka (1955) using BAEE as substrate and the efficacy of PMSF as an irre-

versible inhibitor was confirmed.

3.3.2. Immunological methods

Ouchterlony double diffusion in agar and immunoelectrophoresis were carried out as previously described (chapter 2).

3.3.3. SDS - Polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in 1% SDS was carried out according to the method of Fairbanks et al. (1971). A Shandon SAE-2734 apparatus for polyacrylamide electrophoresis (Shandon Scientific Company, London) fitted with eight borosilicate glass tubes (diameter 5 mm, height 8 cm) was used. Gels containing 4% or 5.6% (w/v) acrylamide and 0.21% crosslinker (BIS) were routinely used. Before gelling had occurred, water was carefully laid over the polyacrylamide solution in the tubes to ensure that the gel surface was flat.

Lyophilysed protein samples were dissolved in 0.01 M-Tris-HCl buffer, pH 8.0, which also contained 0.001 M EDTA, 1% SDS, 10% sucrose and 0.001% Pyronin G (tracking dye). Proteins already in solution in other buffers were dialysed overnight at room temperature against the same SDS-buffer as above. Four to five microlitre samples containing approximately 50 μ g protein were carefully applied to the top of the gel cylinders (already submerged under electrophoresis buffer) by means of a graduated microsyringe (Hamilton Corp., California, U.S.A.). A current of 5 mA/gel was applied for 75 min, during which time the tracking dye migrated to close to the lower (anodal) tip of the gels.

The gel cylinders were removed from their glass tubes by careful rimming with a 3-inch 24-gauge syringe needle whilst submerged under water. They were stained overnight in a 0.025% solution of Coomassie Brilliant Blue dye dissolved in isopropanol - acetic acid - distilled water (25:10:65 v/v). Destaining was a two-step procedure and required the following solutions:

Solution 1: isopropanol - acetic acid - distilled water (10:10:80 v/v)

Solution 2: 10% acetic acid in distilled water.

Staining and destaining took place in a special container made out of a 2 cm (width) x 9 cm (height) x 12.5 cm (length) clear Perspex block into which eight holes (diameter: 9 mm; depth: 8 cm) had been drilled. The free flow of solution around the gel cylinders which were placed in these holes, was ensured by 3 mm wide slots machined vertically on both sides from the bottom to within 5 mm of the top of each hole. The container with gels was submerged in the appropriate solution, which was stirred magnetically. Destaining in solution 1 took place for 7 to 8 h and in solution 2 until the gel cylinders were clear (\pm another 24 h).

3.3.4 Trypsin digestion of normal IgM and IgM(Sad)

3.3.4.1 High temperature (56°C) digestion The method employed for the high temperature trypsin digestion of IgM preparations was essentially that described by Plaut and Tomasi (1970). Solutions (6 mg/ml) of purified normal IgM or IgM(Sad) in 0.05 M-Tris-0.01 M-CaCl₂, pH 8.0, were digested at 56°C with trypsin

for periods ranging from 0.5 h to 4 h. Trypsin was dissolved in a minimum volume of 0.001 N-HCl-0.05 M-NaCl, pH 3.0, before addition to the protein solution, prior dissolution of trypsin in this acidic medium being necessitated by its poor solubility in the pH 8.0, Tris - NaCl buffer in which IgM was normally dissolved. An enzyme-to-protein ratio of 1 : 25 was always used for proteolysis. Digestion was terminated by the slow addition of the trypsin inhibitor PMSF to a hundredfold molar excess. The calculated amount of PMSF to be added was dissolved in a volume of isopropanol such that the final isopropanol concentration in the digest-inhibitor mixture was 10% (v/v). Such aqueous isopropanol solutions were not dialysed before column chromatography commenced and had no obvious detrimental effects on either the packing material or chromatography itself. In fact, detection of the characteristic smell of isopropanol in the effluent served as a good indication that no further peptide peaks would be eluted.

Small-scale experiments on the effect of NaCl concentration and digestion time on the nature and qualitative distribution of tryptic fragments from normal IgM as well as IgM(Sad) were carried out as follows:

A 10-ml digestion mixture was made up as above and two 3-ml aliquots were then pipetted into 10-ml glass bottles containing sufficient solid NaCl to yield solutions 0.5 M and 1.0 M in NaCl respectively. The three digestion mixtures were stoppered and incubated simul-

taneously in a waterbath at 56°C . Digestion was terminated at pre-selected time intervals by withdrawing $225\ \mu\text{l}$ samples and transferring them to test tubes containing $25\ \mu\text{l}$ of PMSF dissolved in isopropanol. Immunoelectrophoretic analysis of the digestive products of these tubes was begun 30 min after the last sample had been taken, and the residual solutions were dialysed overnight for SDS-PAGE analysis.

The influence of the method used to precipitate IgM(Sad) upon subsequent susceptibility to trypsin was investigated by precipitating one half of the Waldenström plasma from a single plasmapheresis with PEG and the other half by dialysis against dilute phosphate buffer. The two precipitates were purified by column chromatography, digested with trypsin and their chromatographic patterns compared.

3.3.4.2 Low temperature (37°C) digestion of IgM(Sad)

Trypsin digestion of IgM(Sad) at 37°C and column chromatographic separation of the products were carried out, according to Miller and Metzger (1966), in order to obtain $\text{F(ab)}_2\mu(\text{Sad})$ for comparative SDS-PAGE analysis.

3.3.5 Column chromatography

One hundred centimetre long glass columns (Pharmacia, Uppsala, Sweden) with diameters of 10 cm, 5 cm and 2.5 cm were used for molecular exclusion chromatography and a 2.5×45 cm glass column (Pharmacia, Uppsala, Sweden) was used for cellulose ion-exchange chromatography. All chromatography columns were fitted with flow adaptors and were

siliconised before use (Siliclad - Clay Adams, New Jersey, U.S.A.). Molecular exclusion chromatography took place under gravity flow and constant hydrostatic pressure, whilst a Beckman Model 141 gradient pump (Beckman Instruments, California, U.S.A.) was used for ion-exchange chromatography. Packing materials for molecular exclusion chromatography included Sepharose 6B, Sephadex G-100 (superfine) and Sephadex G-200.

3.3.6 Antiserum production

Rabbit antisera to the $Fab\mu$ and $Fc_{5\mu}$ -fragments of normal IgM were produced as described in chapter 2. Anti- $Fd\mu$ antiserum was obtained by neutralising the anti-L-chain activity of selected anti- $Fab\mu$ antisera by adsorption with insolubilised IgG (γ_2L_2) until no more antibody with anti-L-chain specificity could be detected (Ouchterlony double diffusion, 1% (w/v) IgG solution as antigen).

3.4 RESULTS

3.4.1 The effect of NaCl concentration on the high temperature trypsin digestion of normal IgM and IgM(Sad)

3.4.1.1 Small-scale experiments. In an effort to obtain maximum yields of the $Fab\mu$ and $Fc_{5\mu}$ fragments of normal IgM and IgM(Sad), optimum digestion conditions were determined in pilot experiments in which both the NaCl molarity and the duration of digestion were varied.

The IE and SDS-PAGE results obtained from one such experiment on normal IgM are presented in Plates 9 and 10 respectively. Although IE results cannot be

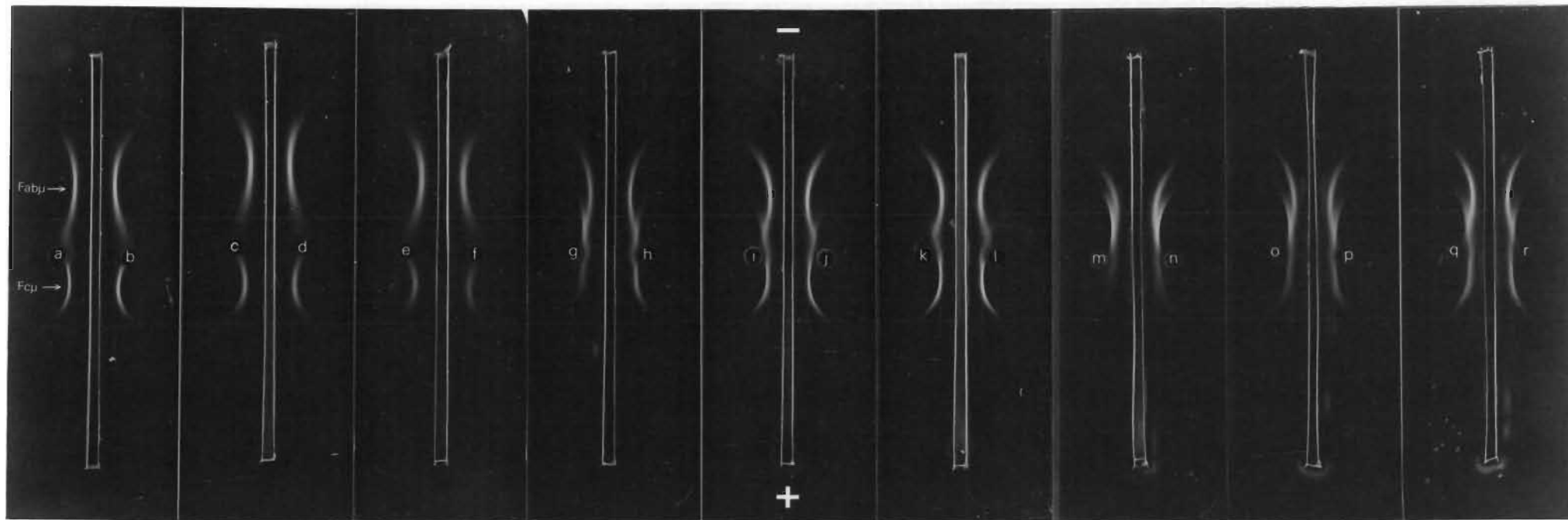


Plate 9

IE analysis of tryptic digests (56°C) of Normal IgM (6 mg/ml in 0.05 M-Tris-0.01 M- CaCl_2 , pH 8.0) obtained under the following conditions:

- a-f: 0.0 M NaCl, 1-6 h digestion (1 h increments);
- g-l: 0.5 M NaCl, 1-6 h digestion (1 h increments);
- m-r: 1.0 M NaCl, 1-6 h digestion (1 h increments).

The troughs contained unadsorbed anti-IgM antiserum.

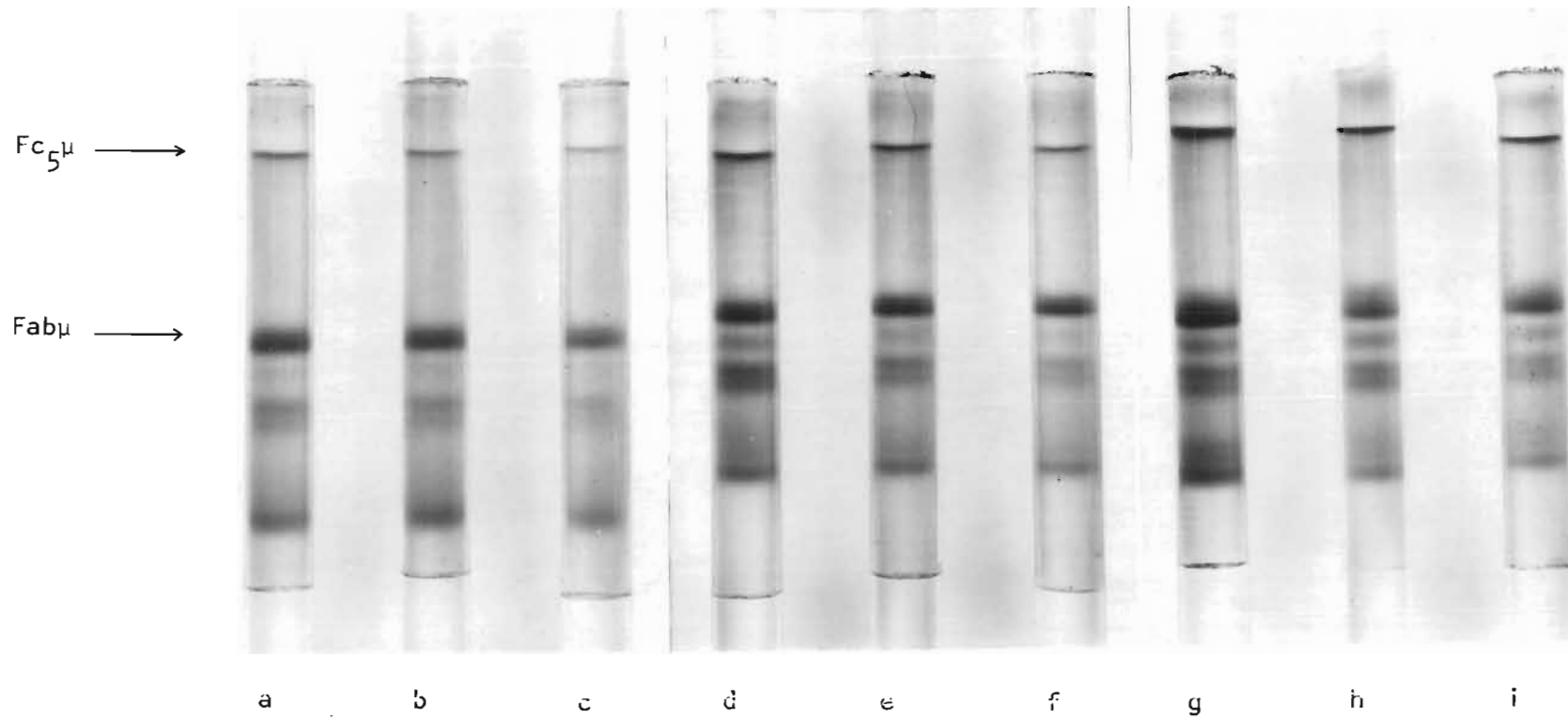


Plate 10

SDS-PAGE (5.6% gel) analysis of tryptic digests (56°C) of Normal IgM (6 mg/ml in 0.05 M-Tris-0.01 M- NaCl_2 , pH 8.0) obtained under the following conditions:

- a-c: 0.0 M NaCl - 2, 4 and 6 h digestion;
- d-f: 0.5 M NaCl - 2, 4 and 6 h digestion;
- g-i: 1.0 M NaCl - 2, 4 and 6 h digestion.

interpreted strictly quantitatively, a decrease in $Fc_{5\mu}$ yield with time when NaCl is absent, is evident from Plate 9 (a - f). Of greater importance, however, is the variation in the precipitin patterns obtained at various NaCl concentrations, but at equivalent digestion times. Comparison, for example, of Plate 9a (0.0 M NaCl, 1 h digestion) and Plate 9g (0.5 M NaCl, 1 h digestion) not only shows that while the $Fc_{5\mu}$ -precipitin line is less distinct in Plate 9g, it also occurs in a more cathodal position. Plate 9g furthermore shows two precipitin lines on its cathodal side (possibly undigested IgM and Fab_{μ}) whereas Plate 9a only has a characteristic Fab_{μ} precipitin line. These observations suggested a slower rate of trypsin digestion of IgM in the presence of NaCl which could have been due to either an inhibition of the enzyme or a stabilization of the protein substrate, IgM, by NaCl (Plate 9m). BAEE assays of trypsin at 56°C , either at zero time or after a 30-min preincubation of the enzyme did not reveal any loss in trypsin activity in the presence of NaCl. Although the BAEE-assay admittedly measures esterolytic and not proteolytic activity, it seems reasonable to assume that the effect of NaCl was not to change trypsin activity, but rather to stabilize IgM against tryptic digestion by an electrolyte-protein interaction. Circumstantial evidence supporting this view may be found in Plate 9r in which it is evident that even at the highest NaCl concentration tested, a sufficiently long

digestion time will eventually lead to IgM fragmentation.

The qualitative results obtained from IE were confirmed by SDS-PAGE analysis (Plate 10) of the same samples. In the absence of purified Fab μ and Fc μ_5 fragments, the multiple bands shown in Plate 10 could not be positively identified. However, it was assumed that the two uppermost bands were Fc μ_5 and Fab μ respectively and that the lower ones were smaller subfragments. It can be concluded that in the absence of NaCl a more rapid fragmentation of normal IgM took place than in its presence.

The IE and SDS-PAGE results obtained for IgM(Sad) from a comparable experiment extending over a 3-h digestion period are shown in Plates 11 and 12 respectively. The results were very similar to those obtained for normal IgM and the same conclusions regarding the protective effect of NaCl can be reached. One obvious difference between these two immunoglobulins was the greater susceptibility of IgM(Sad) to trypsin fragmentation. This is reflected by the shorter digestion periods necessary to obtain complete fragmentation of IgM(Sad). (Compare for instance Plates 11 i and 9 i). Conditions of NaCl concentration and digestion time for large-scale fragmentation of normal IgM and IgM(Sad) were selected on the basis of the above results. Experience showed, however, that this approach did not always give the desired results, since fragment yields quite different from those expected were often obtained

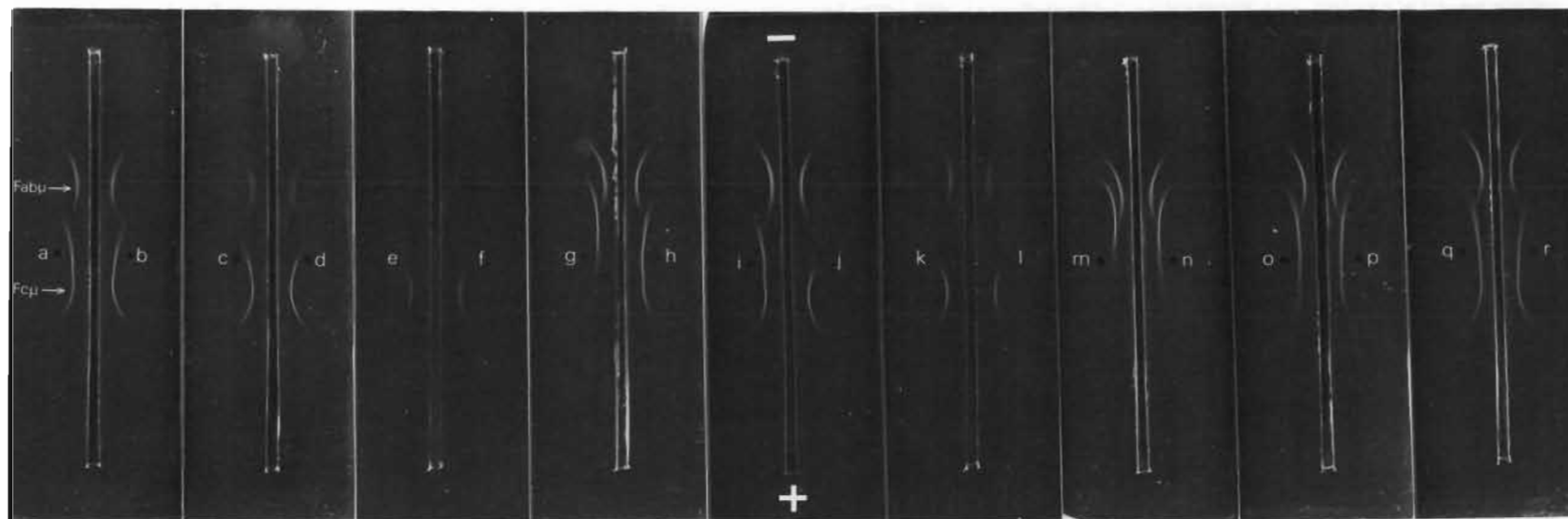


Plate 11

IE analysis of tryptic digests (56°C) of IgM(Sad) (6 mg/ml in 0.05 M-Tris-0.01 M- CaCl_2 , pH 8.0) obtained under the following conditions:

- a-f: 0.0 M NaCl, 0.5-3.0 h digestion (0.5 h increments);
- g-l: 0.5 M NaCl, 0.5-3.0 h digestion (0.5 h increments);
- m-r: 1.0 M NaCl, 0.5-3.0 h digestion (0.5 h increments).

The troughs contained unadsorbed anti-IgM antiserum.

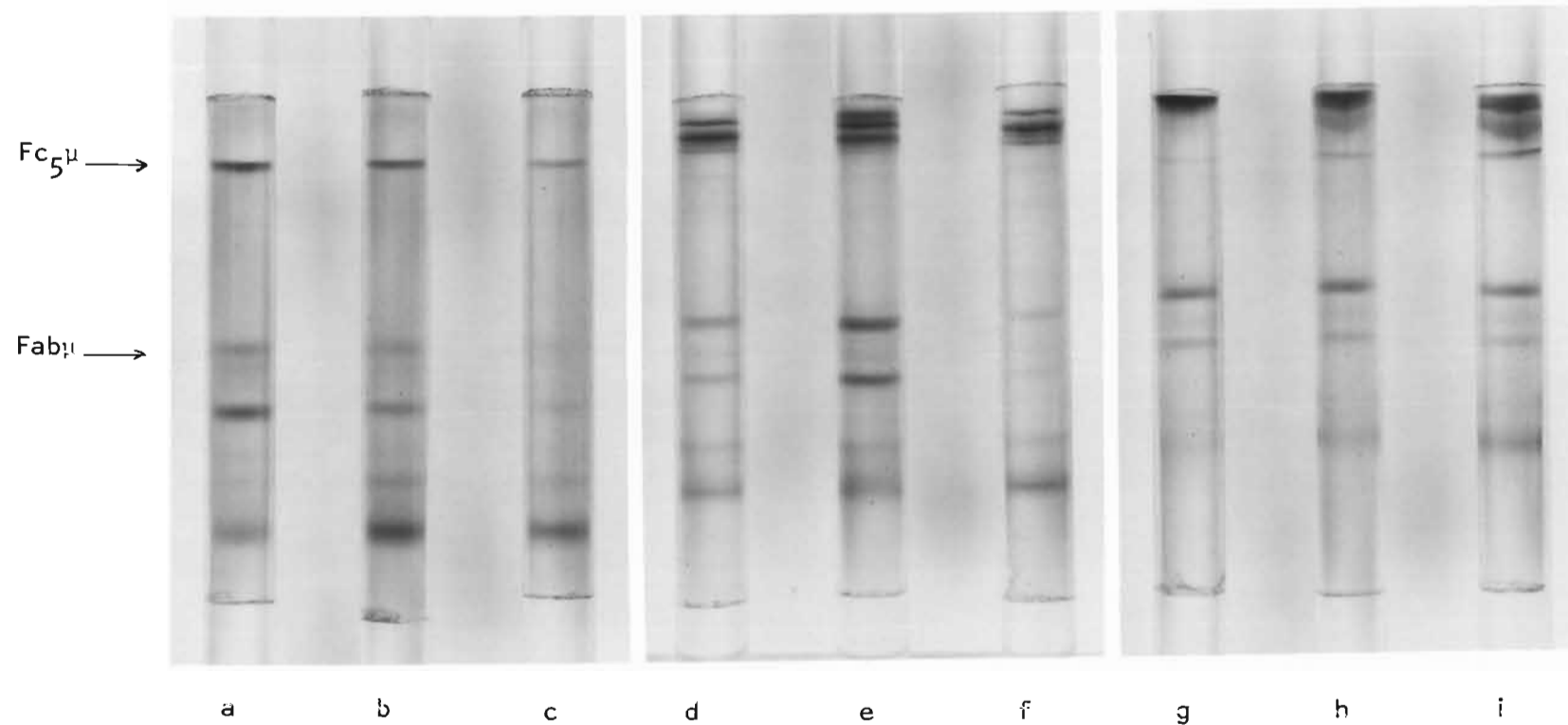


Plate 12

SDS-PAGE (5.6% gel) analysis of tryptic digests (56°C) of IgM(Sad) (6 mg/ml in 0.05 M-Tris-0.01 M- CaCl_2 , pH 8.0) under the following conditions:

- a-c: 0.0 M NaCl - 1, 2 and 3 h digestion;
- d-f: 0.5 M NaCl - 1, 2 and 3 h digestion;
- g-i: 1.0 M NaCl - 1, 2 and 3 h digestion.

from scaled-up digestions.

3.4.1.2 Effect of sodium chloride concentration and digestion time on the yields of tryptic fragments of IgM(Sad) obtained by column chromatography. Optimum conditions for the large-scale tryptic fragmentation of IgM(Sad) proved extraordinarily difficult to extrapolate from pilot experiments and widely disparate results were obtained from small and large-scale digestion of the same IgM(Sad) preparation. Another perplexing observation was that the method of IgM(Sad) precipitation (PEG or euglobulin) had a profound effect on the yield of proteolytic fragments. Fab μ yields of 15.4% and 44.4% were obtained respectively, for instance, in a comparative experiment using IgM(Sad) from a single donation of macroglobulinaemic plasma but precipitated either by PEG or by the euglobulin method. At this stage the cause of this difference in susceptibility to trypsin cleavage can only be speculated upon and is conceivably related to a small, but significant conformational change induced by one method of precipitation, but not by the other. Paul et al. (1971) have found that, for IgM(Ou), a carbohydrate moiety on asparagine 221 lies in close proximity to the trypsin cleavage point, viz. arginine 214 (Fig. 13). They have suggested that the high temperature during digestion may cause sufficient unfolding of the intact molecule to allow a close approach of enzyme to this otherwise sterically blocked part of the peptide chain. The

higher turbidity usually found for redissolved euglobulin precipitated IgM(Sad) could perhaps be taken as an indication that euglobulin precipitation is a harsher technique than PEG precipitation, leading to a greater extent of unfolding (partial denaturation) and eventually to greater sensitivity to trypsin cleavage.

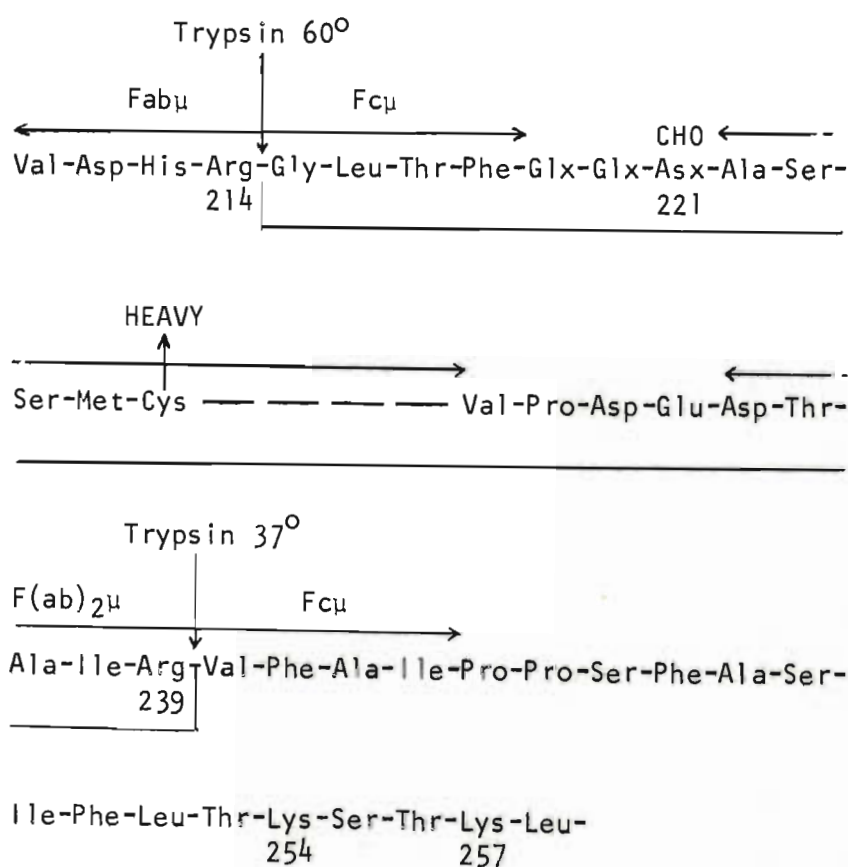


Fig. 13: Amino acid sequence of the hinge region of IgM (0u) (Paul et al., 1971).

Because of the higher yields of Fab_μ and Fc_{5μ} generally obtained from euglobulin precipitated IgM(Sad), a study (column chromatography) of the effect of NaCl concentration and digestion time on fragment yields was undertaken on such preparations. The results of one

experiment are given in Table 1 which shows an expected decrease in the high molecular weight fraction (a) and an increase in small peptides (c) with digestion time. The inhibitory effect of NaCl on the rate of proteolysis of IgM(Sad) is again evident. This is illustrated by a higher content of high molecular weight proteins (fractions a + b) and a lower yield of small peptides (fraction c) at comparable time intervals in the presence of NaCl. Inspection of the percentage yields of fraction c obtained from a 15 min trypsin digest in the absence and presence of 1 M NaCl (Table 1), also shows that NaCl depressed the yield of this fraction by approximately 55%. The results of this experiment were of little value in establishing conditions for the large scale fragmentation of normal IgM and IgM(Sad).

Table 1 Chromatographic yields (%) of tryptic fragments of euglobulin precipitated IgM(Sad) as a function of time of digestion and NaCl concentration. Digests fragmented on Sephadex G-200 with 0.05 M-Tris-0.5 M-NaCl (pH 8.0) as eluant.

Column fractions	NaCl, M	% yields after		
		7.5 min	15 min	30 min
Fraction a	0.0	23.2	14.8	13.2
"	0.5	69.8	37.4	17.9
"	1.0	78.4	60.4	28.7
Fraction b	0.0	16.5	17.5	9.3
"	0.5	a	15.0	21.4
"	1.0	a	a	20.4
Fraction a + b	0.0	39.7	32.3	22.5
"	0.5	69.8	52.4	39.3
"	1.0	78.4	60.4	49.1
Fraction c	0.0	24.9	26.0	9.8
"	0.5	12.1	10.5	8.3
"	1.0	8.0	11.7	11.5

a: No separation into fractions a and b occurred

3.4.2 Large scale trypsin digestion of normal IgM and separation of the Fab μ and Fc $_5\mu$ -fragments

In this experiment, a solution of 677 mg of purified normal IgM in 111 ml of 0.05 M-Tris-0.01 M-CaCl $_2$ buffer, pH 8.0, containing 0.5 M NaCl was digested for 4 h at 56°C. Column chromatography on Sephadex G-100 resolved the digest into three peaks as shown in Fig. 14. Fraction 14c consisted of dialysable peptides. Immuno-electrophoretic and SDS-PAGE analyses of fractions 14a and 14b (Plates 13 and 14) showed that these two fractions represented, respectively, Fc $_5\mu$ and Fab μ -containing material.

This conclusion is based on the anodal and cathodal mobilities of these fragments and their known difference in molecular weight (Plaut and Tomasi, 1970). The analyses of fraction 14a also showed that it was contaminated with undigested IgM.

The presence of an additional faint precipitin line more anodal than that of Fc $_5\mu$ (Plate 13a) was unexpected and the author is unaware of any literature reference to the identity of this fragment. It is possible that this fast anodal component (FAC) is a unique fragment of normal IgM since it was not observed in digests of IgM(Sad).

3.4.3 Purification of normal Fc $_5\mu$

The obvious heterogeneity of fraction 14a illustrated by IE and SDS-PAGE analysis (Plates 13 and 14) necessitated further purification of its Fc $_5\mu$ -component before any physico-chemical characterization of this fragment could be

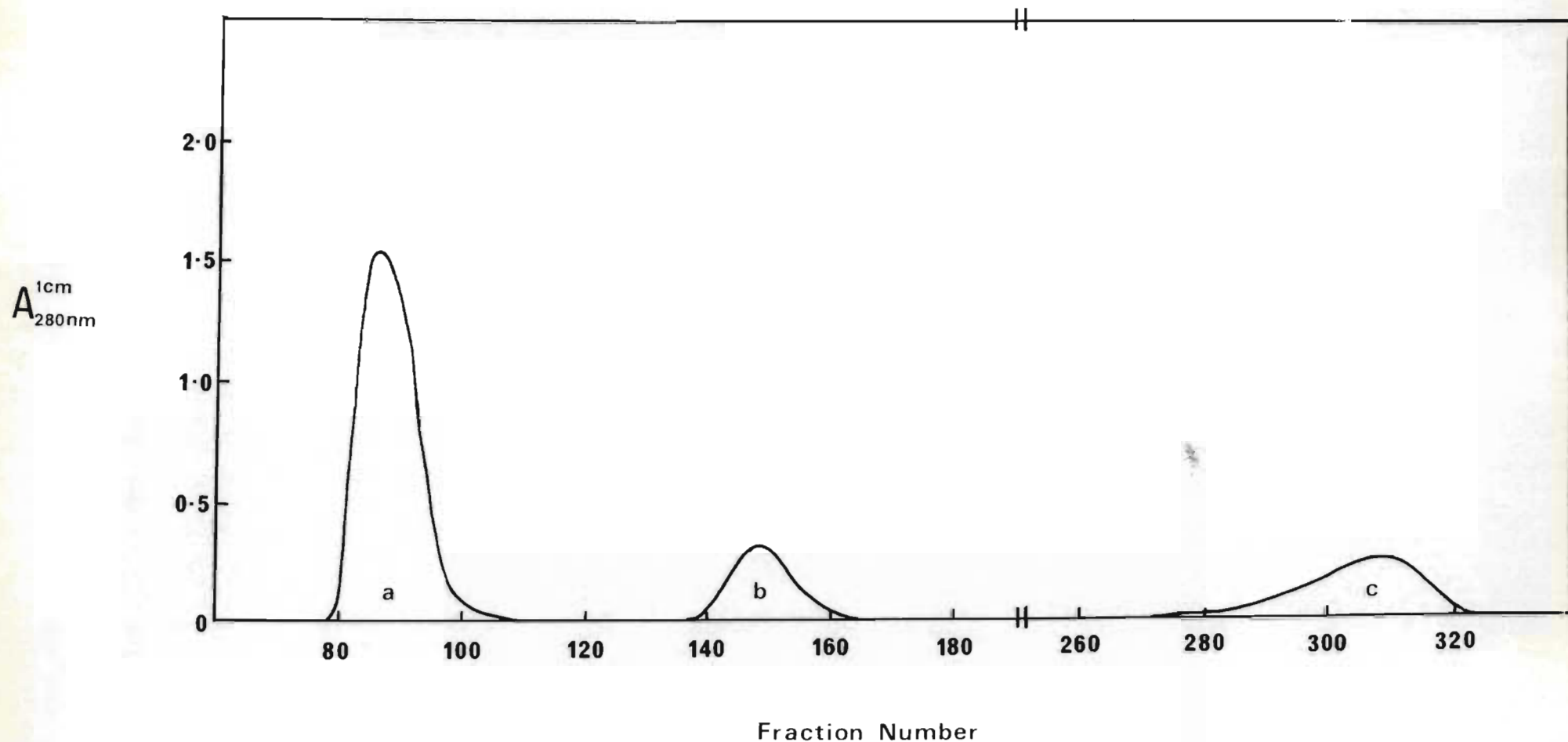


Fig. 14: Sephadex G-100 chromatogram of a 4 h tryptic digest at 56°C of 677 mg normal IgM in the presence of 0.5 M NaCl (10 x 95 cm column; buffer, 0.05 M-Tris-0.5 M-NaCl, pH 8.0; flowrate, 200 ml/h; 25 ml fractions).

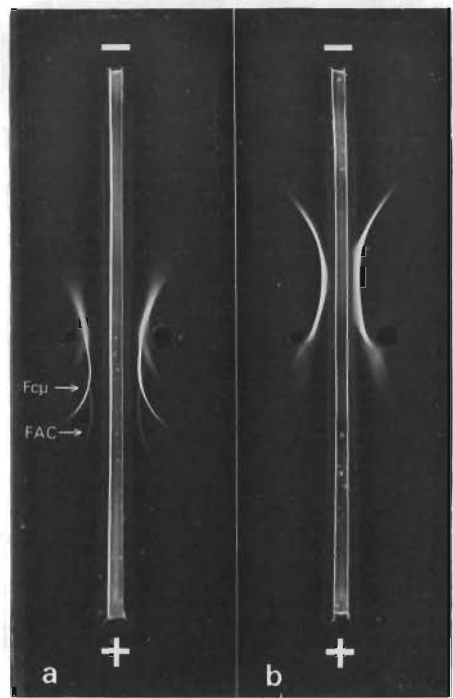


Plate 13 IE analysis of fractions 14a and 14b (Fig. 14) against unadsorbed anti-IgM antiserum.

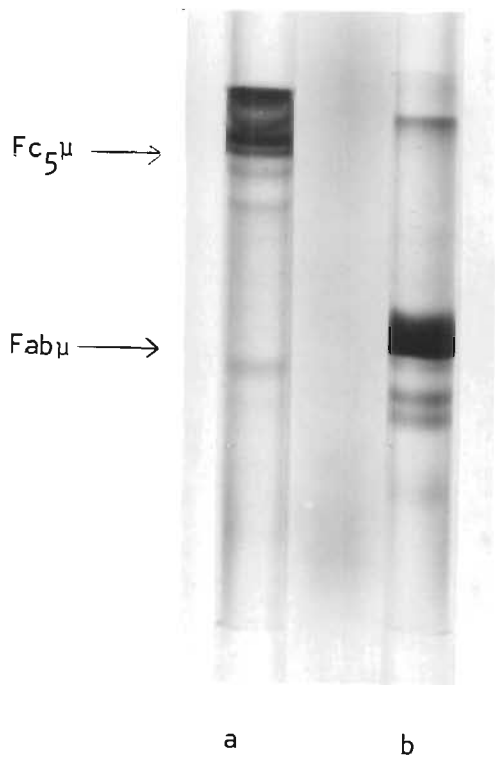


Plate 14 SDS-PAGE (5.6% gel) analysis of fractions 14a and 14b (Fig. 14).

attempted. Immuno-electrophoretic and SDS-PAGE analysis of fractions 15a, 15b and 15c, obtained after chromatography of fraction 14a on Sephadex G-200 (Fig. 15), are depicted in Plates 15 and 16 respectively. When compared with the results for fraction 14a, it is clear that fraction 15b represents a substantially better preparation of $Fc_{5\mu}$, although it is still not homogeneous. Fraction 15a appears to consist of higher molecular weight material (no gel penetration in Plate 16a), comparable to incompletely digested IgM.

The low yield and heterogeneity (Plate 16c) of fraction 15c (FAC) made further attempts at its purification unattractive. An amino acid analysis (Chapter 5) of FAC revealed a relatively high content (26%) of aspartic plus glutamic acid which explains its fast anodal mobility. Its molecular size and anodal position probably rule out the possibility that it is related to the "Int-L" fragment described by Plaut et al. (1972), or J-chain isolated from IgM (Mestecky et al., 1971).

Fraction 15b was finally chromatographed under dissociating conditions in 0.01 M-Tris-4.0 M-GuHCl, pH 8.0, on agarose beads (Sephadex 6B). The slightly asymmetric peak (Fig. 16) represented the only u.v.-absorbing material eluted from this column and was cut into three fractions. SDS-PAGE analyses of fractions 16a, 16b and 16c (Plate 17) suggested that 16b was the most promising in terms of $Fc_{5\mu}$ yield and the absence of higher molecular weight material; its IE (Plate 18) shows the presence of $Fc_{5\mu}$ only. It is neverthe-

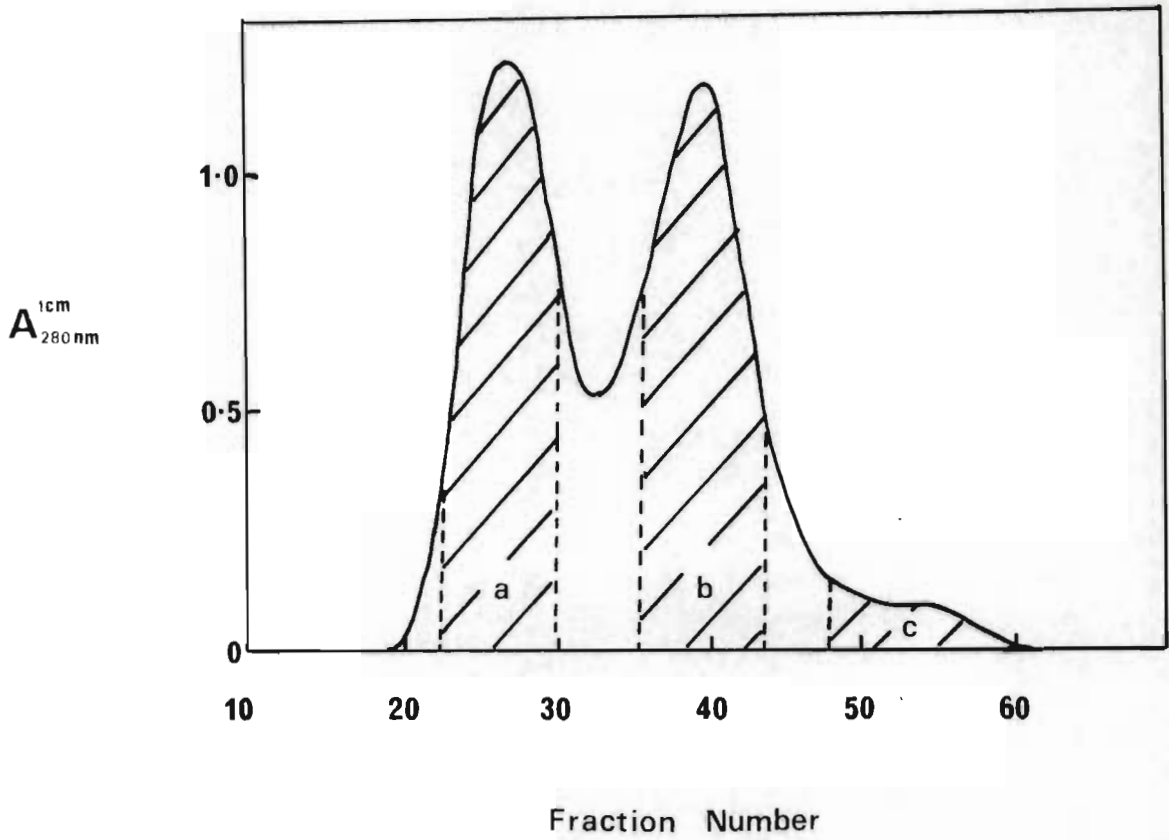


Fig. 15: Elution pattern obtained on rechromatography of 111 mg of fraction 14a (normal $\text{Fc}_{5\mu}$) on a 2.5 x 90 cm column of Sephadex G-200 (buffer, 0.05 M-Tris-0.5 M-NaCl, pH 8.0; flowrate, 20 ml/h; 5 ml fractions).

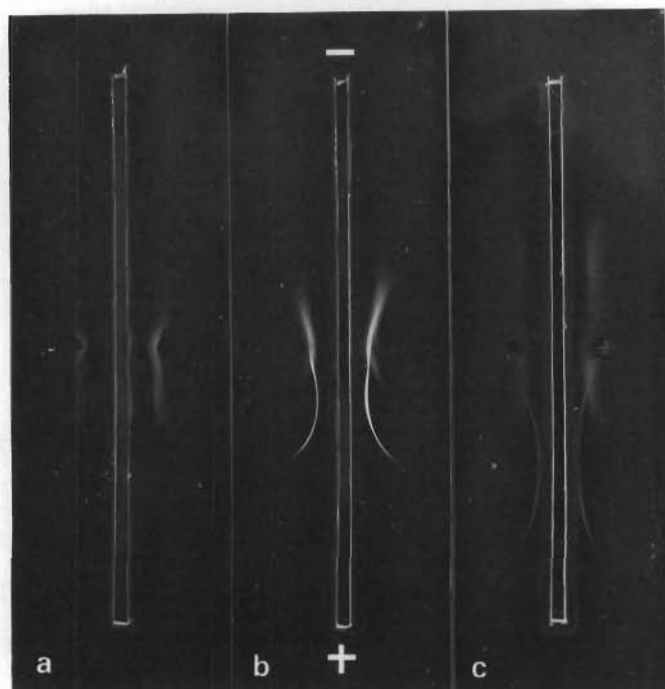


Plate 15 IE analysis of fractions 15a, 15b and 15c (Fig. 15) against unadsorbed anti-IgM antiserum.

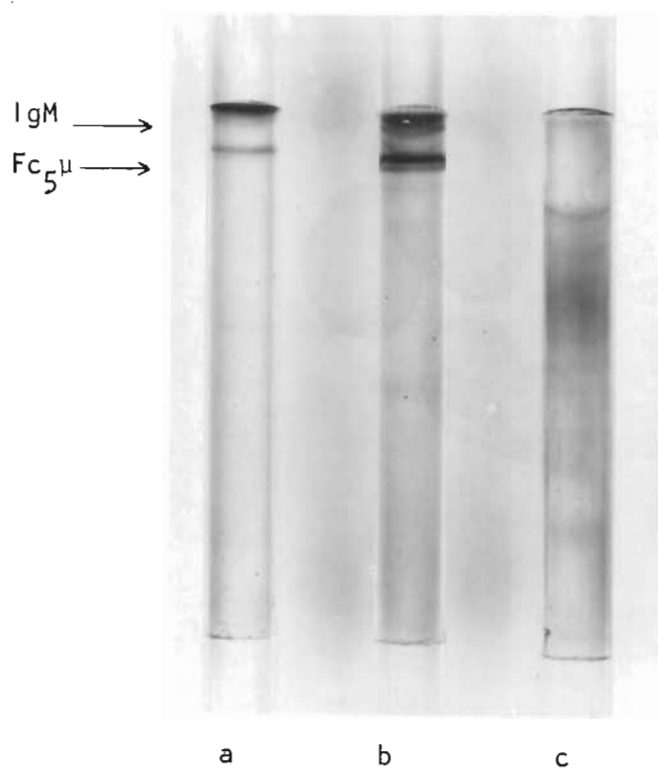


Plate 16 SDS-PAGE (5.6% gel) analysis of fractions 15a, 15b and 15c (Fig. 15)

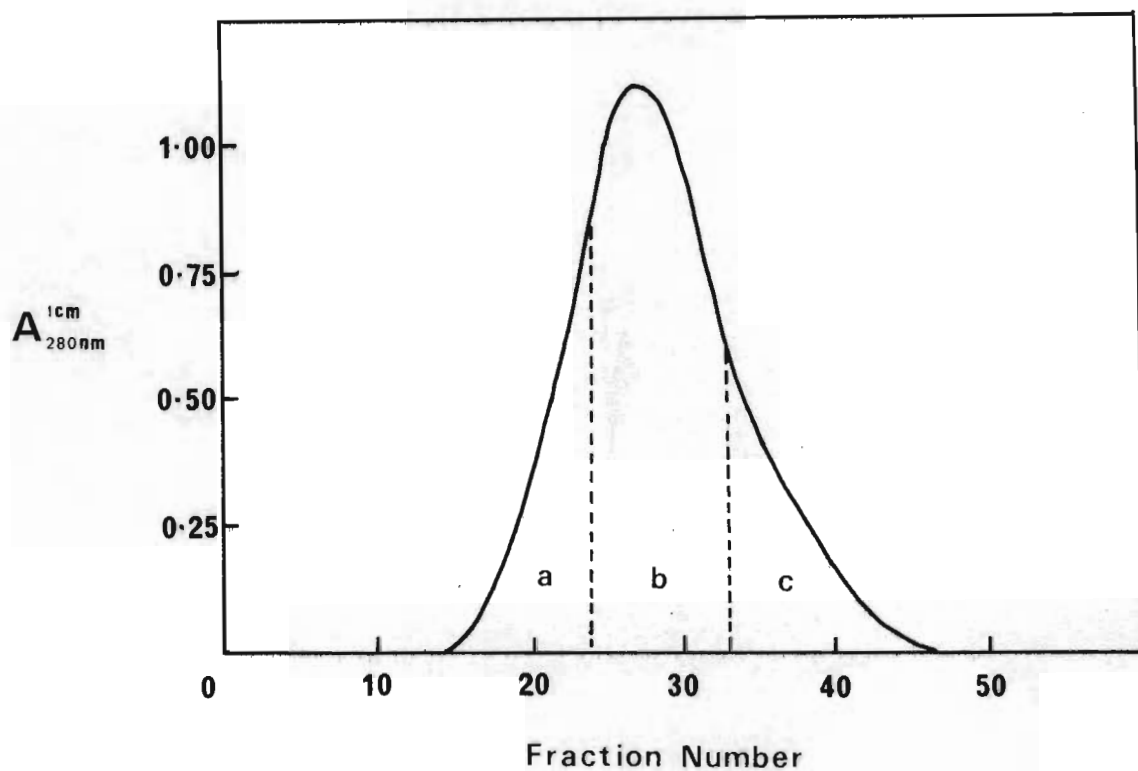


Fig. 16: Elution pattern obtained on rechromatography of 52 mg fraction 15b (normal $\text{Fc}_{5\mu}$) on a 2.5 x 95 cm column of Sepharose 6B (buffer, 0.01 M-Tris-4.0 M-GuHCl, pH 8.0; flow-rate, 10 ml/h; 5 ml fractions).

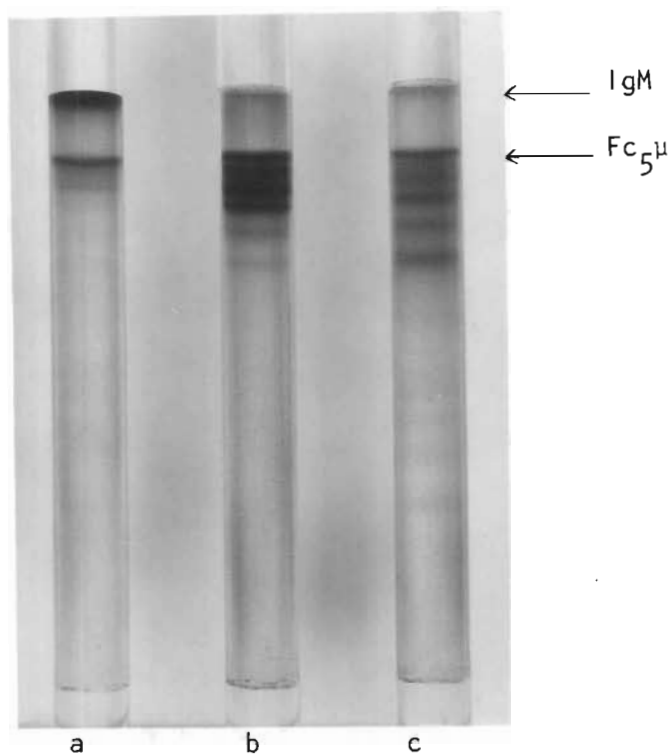


Plate 17 SDS-PAGE (4.0% gel) analysis of fractions 16a, 16b and 16c (Fig. 16, normal Fc₅μ).



Plate 18 IE analysis of fraction 16b against unadsorbed anti-IgM anti-serum.

less clear that even though this fraction appeared to be $Fc_{5\mu}$ of acceptable immunogenic purity, it was still rather heterogeneous as far as molecular size was concerned. However, no purification could be anticipated from the further application of molecular exclusion chromatography techniques.

3.4.4 Purification of normal Fab_{μ}

Porter (1959) made successful use of CM- and DEAE-cellulose chromatography for the separation of the Fab and Fc-fragments of rabbit IgG. Although the IE and SDS-PAGE analysis (Plates 13b and 14b) of the Fab_{μ} -fragment obtained after Sephadex G-100 chromatography of a normal IgM digest (Fig. 14, fraction b) showed a high degree of purity, this material was subjected to DEAE-cellulose chromatography. The elution pattern in Fig. 17 showed two peaks (17a and 17c) with appreciable tailing of 17a. At the time the finding of two peaks rather than one was unexpected. The IE and SDS-PAGE analyses of fractions 17a, 17b and 17c clearly indicate that all three fractions consisted of Fab_{μ} of about the same molecular size (Plate 20), but having cathodal electrophoretic mobilities which decreased from 17a to 17c (Plate 19). The charge difference of these fractions was also borne out by subsequent amino acid analysis (Chapter 5) which showed a higher content of the negatively charged amino acids aspartic and glutamic acid for fraction 17c than for fraction 17a.

The possibility that this result was due to a separation of $Fd_{\mu\kappa}$ and $Fd_{\mu\lambda}$ -species was ruled out by the qualitative presence of both these L-chain types in all fractions (Plate 21).

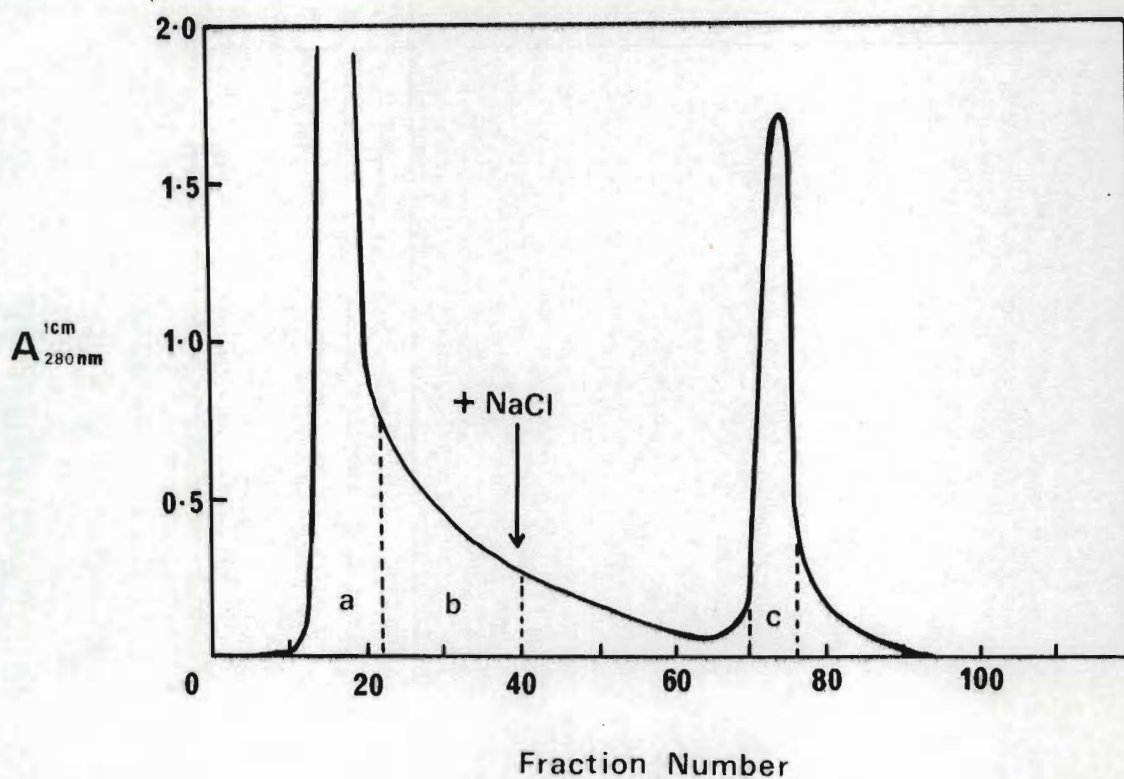


Fig. 17: Elution pattern obtained on rechromatography of 116 mg fraction 14b (normal Fabu) on DEAE-cellulose. (Starting buffer, 0.01 M-phosphate, pH 8.0; gradient to +0.5 M-NaCl; flowrate, 40 ml/h; 5 ml fractions).

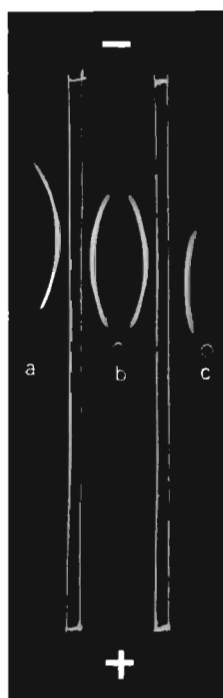


Plate 19 IE analysis of fractions 17a, 17b and 17c (Fig. 17) against unadsorbed anti-IgM antiserum.

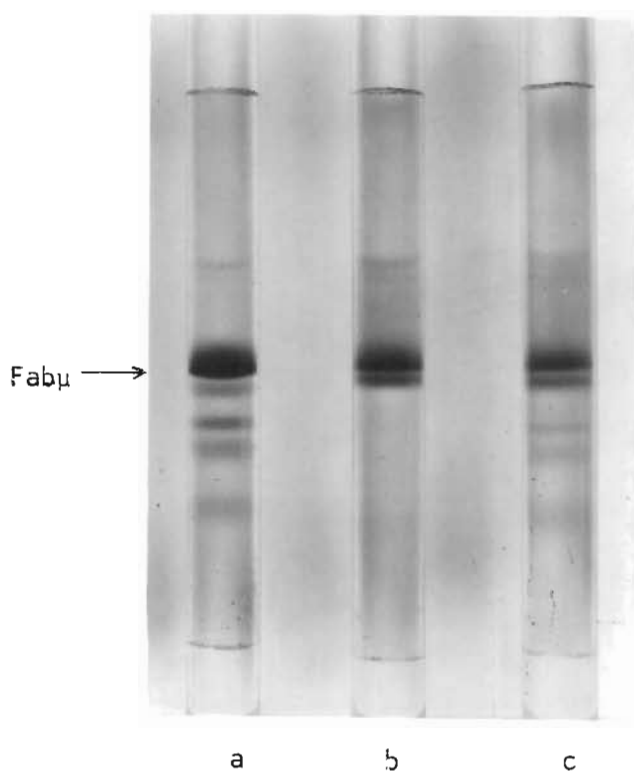


Plate 20 SDS-PAGE (5.6% gel) analysis of fractions 17a, 17b and 17c (Fig. 17).

The origin of the closely-spaced double bands found at the Fab position after SDS-PAGE analysis (Plate 20) is not known. Davie and Osterland (1968) have found that Waldenström IgM's from different patients may be subdivided into two classes, depending on their carbohydrate content. These authors have determined that carbohydrate residues are located on the Fc μ , Fd μ and L-chain peptides and have shown that the carbohydrate moieties of these peptides are all of higher molecular weight in group I than in group II IgM. The glycopeptide with the highest molecular weight is thought to originate from the Fc μ -fragment. If similar considerations apply to the mixed population of normal IgM's, it is doubtful whether the relatively small molecular weight difference between the L-chain plus Fd μ carbohydrate moieties of group I and group II IgM (calculated to be 562 daltons in the case of Waldenström IgM's) would be sufficient to cause the observed double bands for Fab μ . These bands also did not represent subclasses of IgM since antisera made against either fraction 17a or 17c cross-reacted with equal facility with the other fraction after adsorption of anti-L-chain antibodies.

In view of the unusual banding patterns of fractions 17b and 17c, it was decided to concentrate on fraction 17a. As a final purification step, it was subjected to molecular exclusion chromatography on Sephadex G-200 in the presence of guanidine hydrochloride. The middle portion of the only major peak (Fig. 18) was recovered for subsequent molecular weight determination (Chapter 5). The SDS-PAGE pattern of

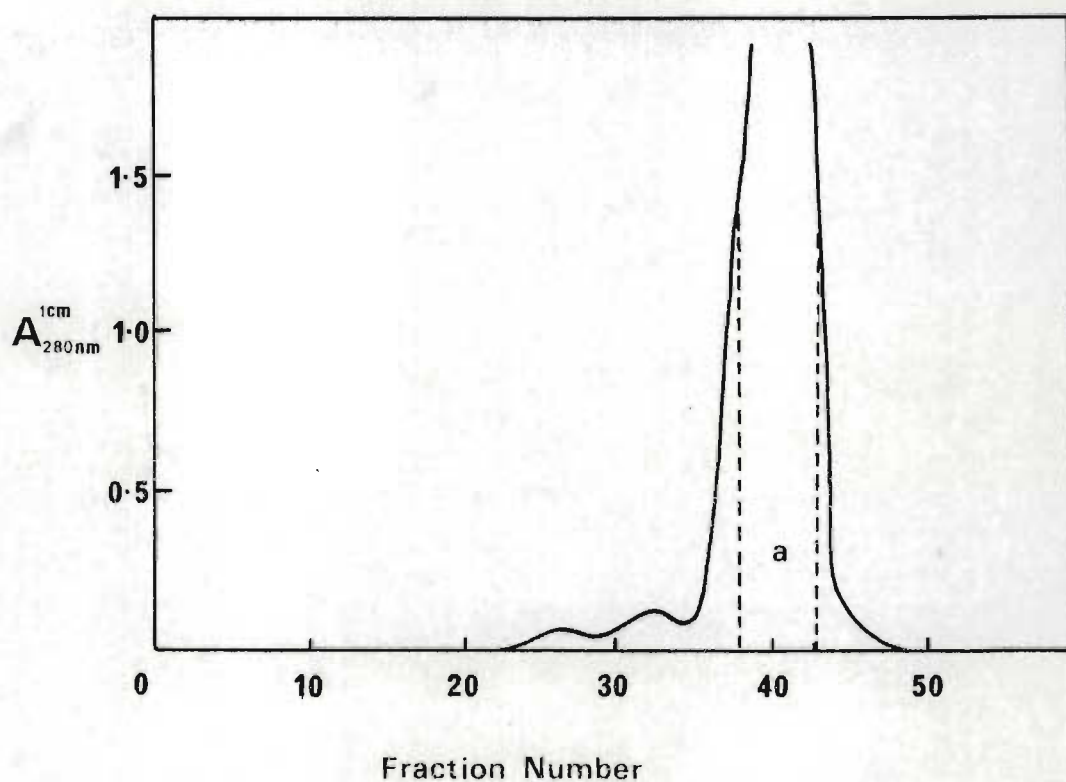


Fig. 18: Rechromatography of 42 mg fraction 17a (normal Fab μ) on a 2.5 x 90 cm column of Sephadex G-200 (buffer, 0.01 M-Tris-4.0 M-GuHCl, pH 8.0; flowrate 5 ml/h; 5 ml fractions).



Plate 21 Ouchterlony double diffusion analysis of fractions 17a, 17b and 17c (Fig.17) against monospecific antisera to κ (K) and λ -chains (L).

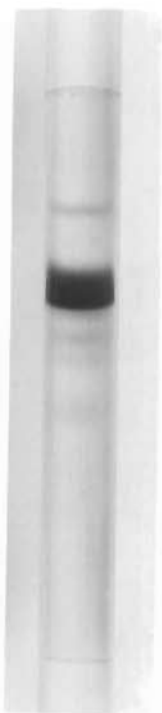


Plate 22 SDS-PAGE (5.6% gel) analysis of fraction 18a (Fig. 18).

this fraction indicated that a high degree of purification had been achieved (Plate 22).

The final yields of Fab_μ and $\text{Fc}_{5\mu}$ -fragments from normal IgM digests were variable and in general, rather low. The prime objective was, however, to obtain samples of sufficient purity for further characterization.

3.4.5 Large scale trypsin digestion of IgM(Sad) and separation of its Fab_μ and $\text{Fc}_{5\mu}$ -fragments

The elution curve shown in Fig. 19 was obtained upon Sephadex G-100 chromatography of a 2-h trypsin digest of IgM(Sad) in the presence of 0.5 M NaCl. Compared with Fig. 14 for normal IgM, IE and SDS-PAGE analysis showed that fractions 19a, 19b and 19c respectively contained $\text{Fc}_{5\mu}$, Fab_μ and a homogeneous low molecular weight peptide (Plates 23 and 24). The molecular size-heterogeneity of the $\text{Fc}_{5\mu}$ fraction is especially noteworthy. The relatively large size of the small peptide-peak (fraction 19f) suggests extensive degradation of part of the IgM(Sad) molecule by trypsin, in contrast to the low yield of such peptides reported by Plaut and Tomasi (1970) on another Waldenström IgM.

3.4.6 Purification of $\text{Fc}_{5\mu}$ (Sad)

The $\text{Fc}_{5\mu}$ fraction (Fig. 19a) obtained above was re-chromatographed on Sepharose 6B (Fig. 20) which gave an elution profile very similar to that observed for normal $\text{Fc}_{5\mu}$ under similar conditions of chromatography (Fig. 16). The same applies to the SDS-PAGE patterns (c.f. Plates 25 and 17) and the IE analysis (c.f. Plates 26 and 18). These results underline the fact that a single sharp precipitin

1cm
280nm

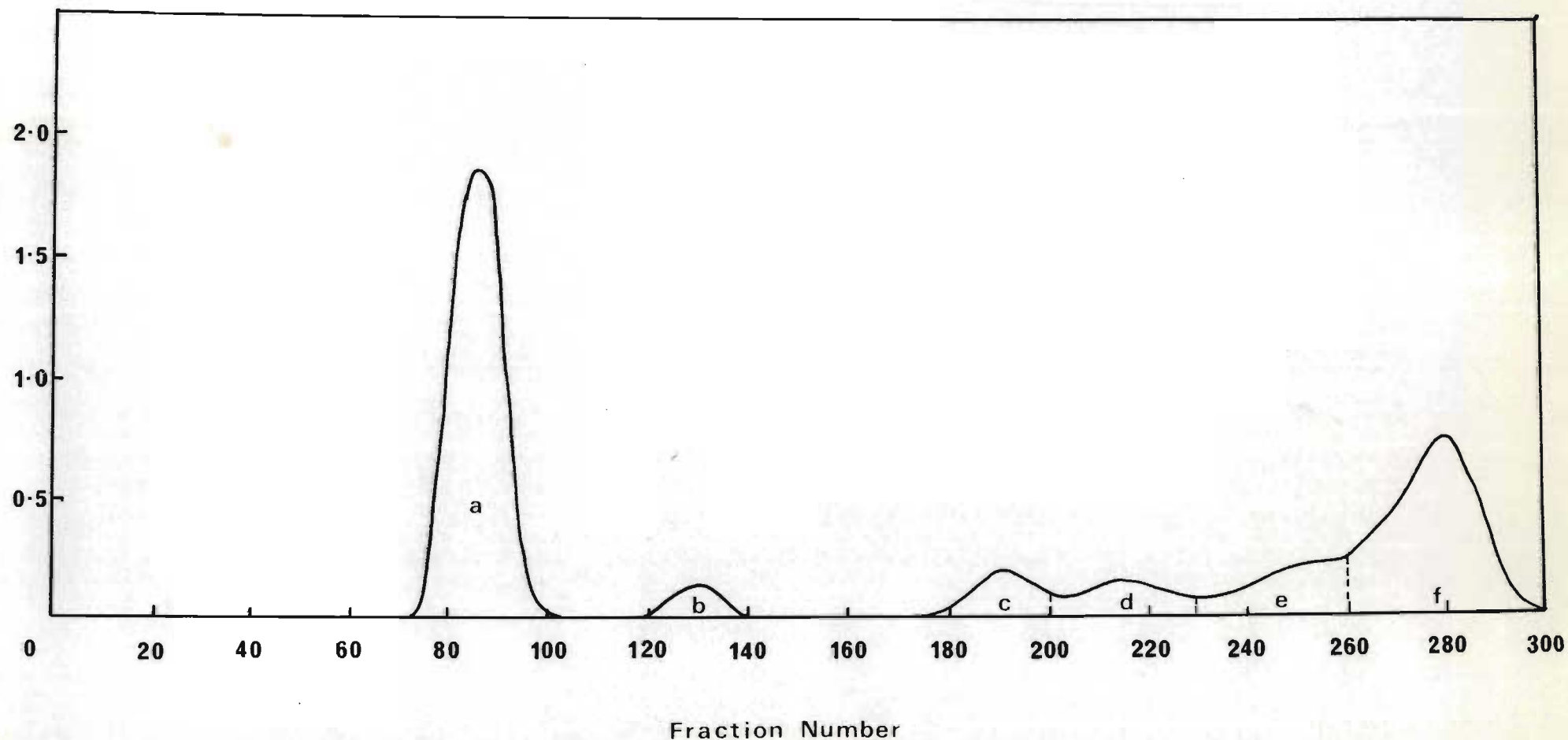


Fig. 19: Chromatogram of tryptic digest of 1200 mg IgM(Sad) on a 10 x 95 cm column packed with Sephadex G-100 (buffer, 0.05 M-Tris-0.5 M-NaCl, pH 8.0; flowrate 200 ml/h; 25 ml fractions).

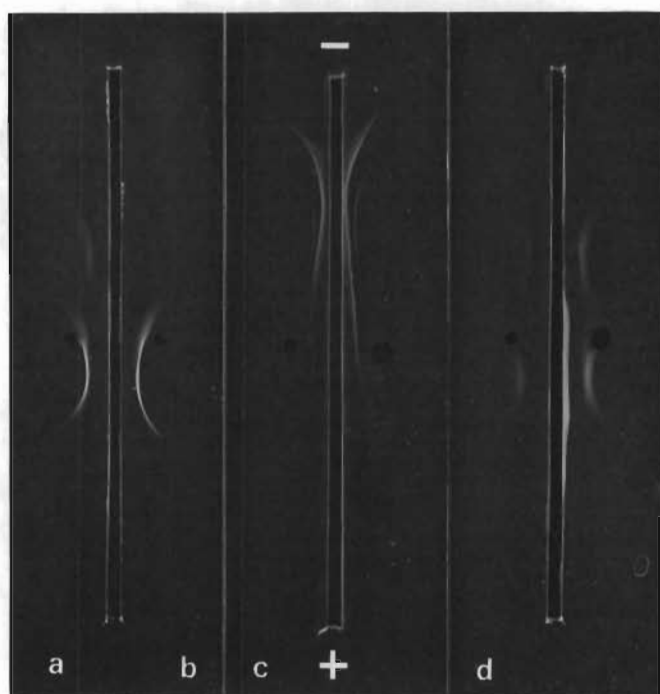


Plate 23 IE analysis of (from left to right) IgM(Sad) tryptic digest, fractions 19a, 19b and 19c (Fig. 19) against unadsorbed anti-IgM antiserum.

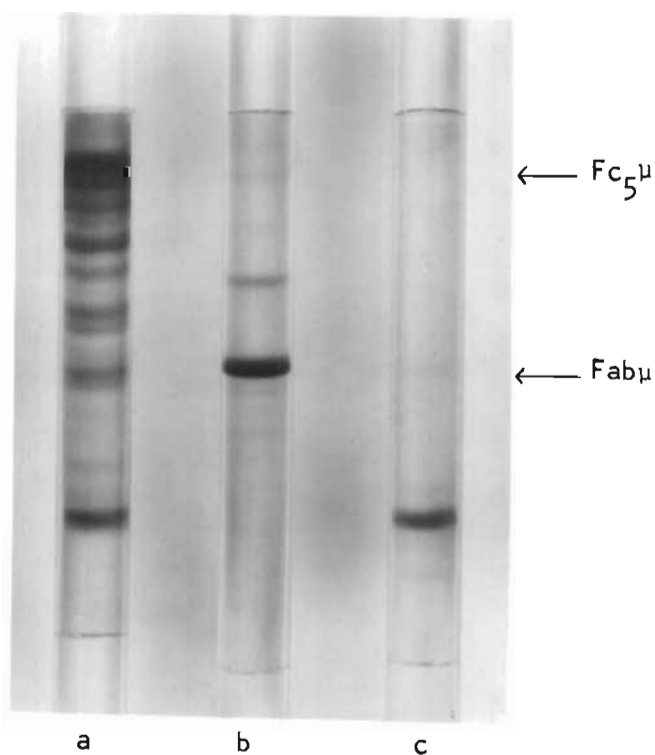


Plate 24 SDS-PAGE (5.6% gel) analysis of fractions 19a, 19b and 19c (Fig. 19).

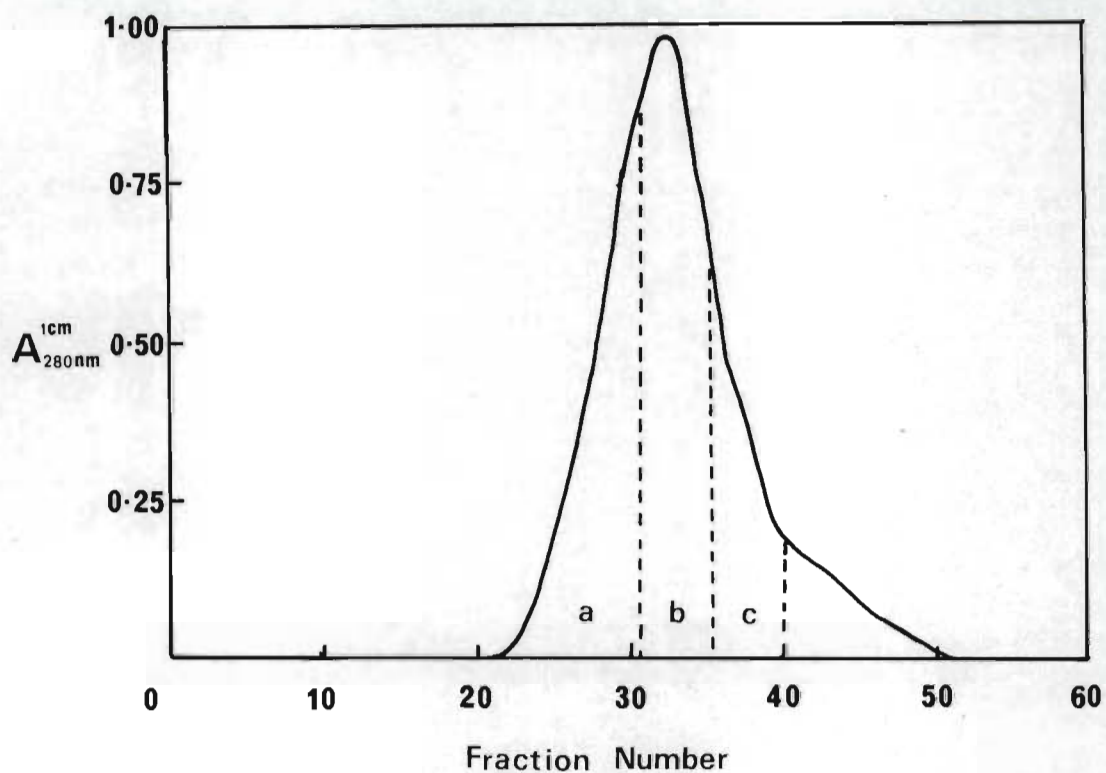


Fig. 20: Rechromatography of 80 mg fraction 19a (Fc₅uSad) on a 2.5 x 95 cm column packed with Sepharose 6B (buffer, 0.01 M-Tris-4.0 M-GuHCl, pH 8.0; flowrate, 10 ml/h; 5 ml fractions).

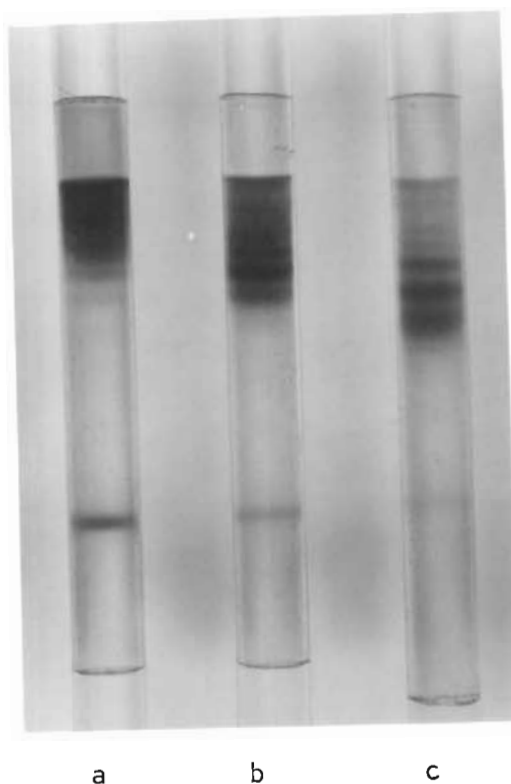


Plate 25 SDS-PAGE (4.0% gel) analysis of fractions 20a, 20b and 20c (Fig. 20).



Plate 26 IE analysis of fraction 20b (Fig. 20) against unadsorbed anti-IgM antiserum.

band (Plate 26) does not necessarily imply size homogeneity (Plate 25b). No further purification of $Fc_{5\mu}(Sad)$ was attempted.

Of interest was the persistence of a small molecular weight component (Plate 25a) with similar mobility in SDS-PAGE as that observed earlier for fraction 19c (Plate 24c). No similar component was found in the digests of normal IgM. A comparison of this component (fraction 19c) by SDS-PAGE analysis with λ -chain, isolated from IgM(Sad), revealed that it had a smaller molecular weight than light chain (Plate 27). Ouchterlony double diffusion against monospecific anti- Fd_{μ} gave no precipitin arc, but anti- $Fc_{5\mu}$ gave a positive result (Plate 28). Component 19c was therefore derived from the Fc-region of the μ -chain of IgM(Sad).

Comparison of the amino acid analyses of fraction 19c and purified $Fc_{\mu}(Sad)$ (Chapter 5) showed a poor correlation, especially with respect to serine, glycine and half-cystine contents. The concentration of half-cystine, relative to leucine, for instance, was approximately five times greater in fraction 19c than in $Fc_{\mu}(Sad)$. Inspection of the primary structure of IgM(0u) (Putnam et al., 1972), revealed that the N-terminal half of $Fc_{\mu}(0u)$ was especially rich in half-cystine residues involved in intra-chain, inter-chain and inter-subunit disulphide linkages (Fig. 22, chapter 4). It is therefore quite plausible that fraction 19c represented the amino-terminal half of the Fc_{μ} -fragment of IgM(Sad). A similar fragment of IgG has been characterized (Turner and Bennich, 1968).

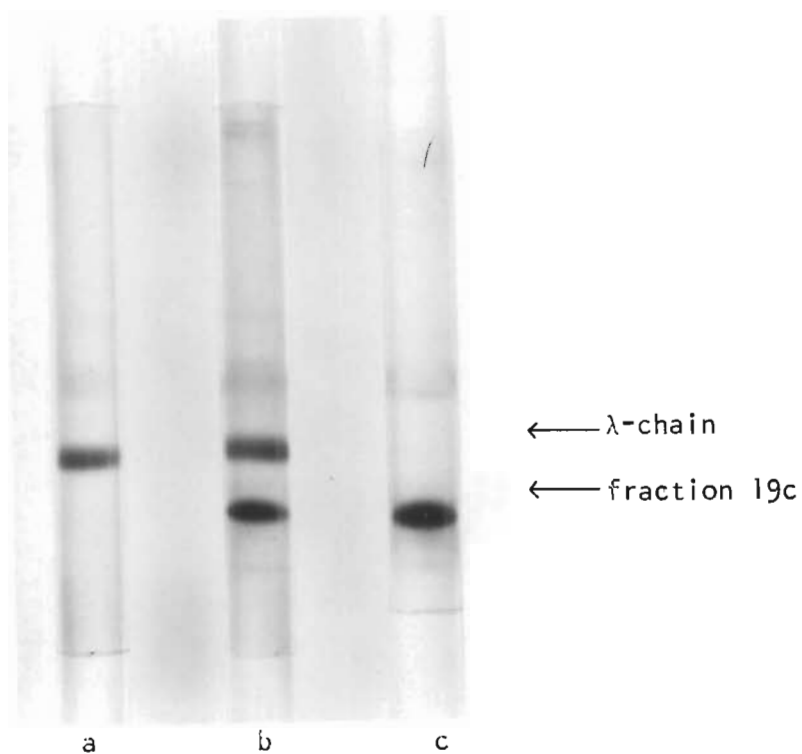


Plate 27: SDS-PAGE (5.6% gel analysis of:

- (a) λ-chain
- (b) mixture of fraction 19c and λ-chain
- (c) fraction 19c (Fig. 19).

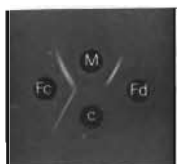


Plate 28: Ouchterlony double diffusion analysis of fraction 19c (bottom well); and purified IgM(Sad) against monospecific anti-Fc₅μ(Fc) and Fdμ(Fd) antisera.

3.4.7 Purification of Fab μ (Sad)

Rechromatography of Fab μ (Sad) (fraction 19b) on DEAE-cellulose gave a single peak (Fig. 21) and the addition of 0.5 M-NaCl to the buffer at the position indicated (arrow) did not elute a second peak as was the case for normal Fab μ (Fig.17). No purification was introduced by this step, as judged by its IE and SDS-PAGE patterns, which were identical to those in Plates 23c and 24b.

An effort was made to establish the identity of the faint upper band (Plate 24b), suspected to be F(ab) $_2\mu$ (Sad), by comparative SDS-PAGE analysis of the following preparations:

F(ab) $_2\mu$ obtained from a 37°C tryptic digest of IgM(Sad); fraction 21 (see above); Fab μ isolated from a 37°C tryptic digest of IgM(Sad); reduced and alkylated IgM(Sad); and Fab μ isolated from IgM(Sad) after trypsin digestion at 56°C for 0.5 h in the presence of 1.0 M NaCl.

The results of this comparative study are shown in Plate 29 and the IE analysis of the F(ab) $_2\mu$ (Sad) and Fab μ (Sad)-fragments obtained from a 37°C tryptic digestion in Plate 30.

The extraordinary diversity of the tryptic Fab μ (Sad)-fragments in comparison with those from normal IgM (Plate 14b) is evident. The triple bands observed for F(ab) $_2\mu$ (Sad) and 37°C-Fab μ (Sad) (Plate 29a and c respectively) were not ideal for identification purposes, but the exact coincidence in mobility of the main F(ab) $_2\mu$ (Sad) band with the faint, upper band in fraction 21a (Plate 29b), was taken as sufficient evidence for identity. It also helped to identify the major bands

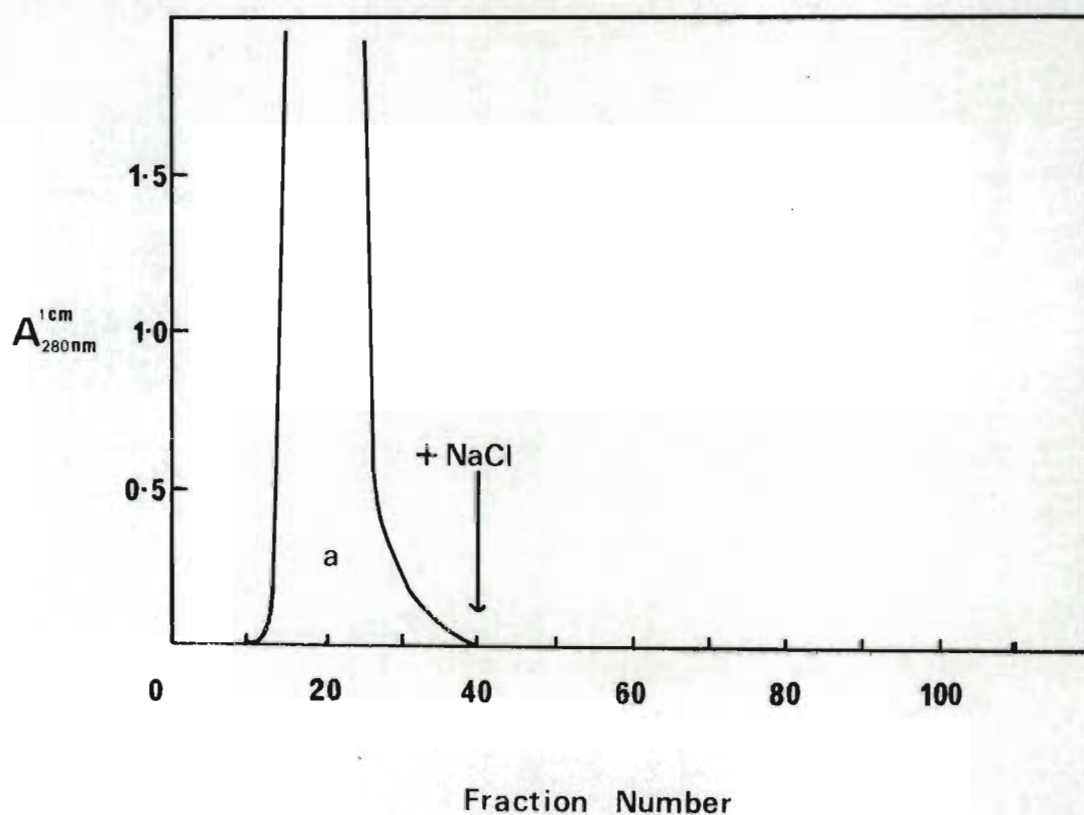


Fig. 21: Rechromatography of 50 mg Fabu(Sad) (fraction 19b) on DEAE-cellulose. (Starting buffer, 0.01 M-phosphate, pH 8.0; gradient to +0.5 M-NaCl; flowrate, 40 ml/h; 5ml fractions).

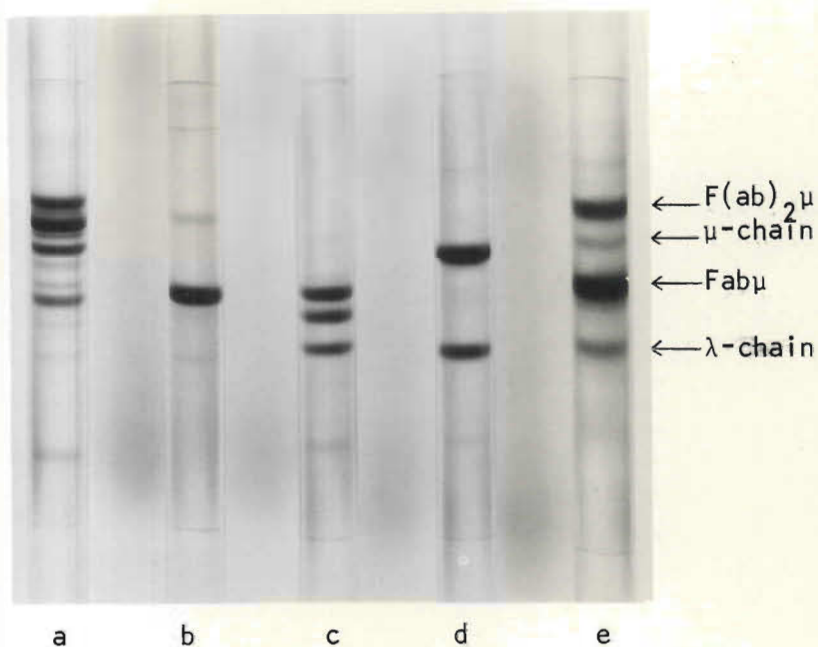


Plate 29 SDS-PAGE (5.6% gel) analysis of:

- (a) $F(ab)_2\mu$ from 37°C trypsin digest of IgM(Sad);
- (b) fraction 21a;
- (c) $Fab\mu$ from 37°C trypsin digest of IgM(Sad);
- (d) reduced and alkylated IgM(Sad);
- (e) $Fab\mu$ from 56°C trypsin digest of IgM(Sad) (1.0 M NaCl - 0.5 h).

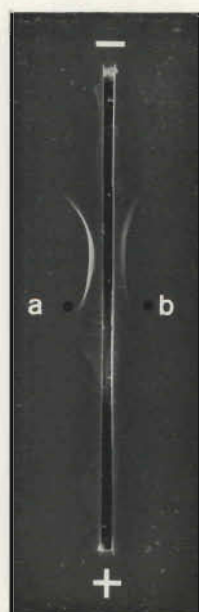


Plate 30 IE analysis of $F(ab)_2\mu$ (a) and $Fab\mu$ (b) from a 37°C trypsin digest of IgM(Sad). Developed against unadsorbed anti-IgM antiserum.

obtained from a NaCl "suppressed" high temperature digestion of IgM(Sad) as $F(ab)_2\mu$ and $Fab\mu$ (Plate 29e). The presence of bands with the same mobilities as those of μ and λ -chain in some of these preparations (see for example Plate 29a, c and e) further served to strengthen the general impression gained from these experiments, namely that it is risky, if not incorrect, to interpret a single sharp precipitin band as a sufficient indication of molecular homogeneity.

3.5 DISCUSSION AND CONCLUSIONS

The application of enzymic fragmentation methods has played a very significant role over the years in the elucidation of the structure and properties of proteins. The work of Ryle et al. (1955) on insulin and Smyth, Moore and Stein (1963) on ribonuclease are well-known examples as far as relatively small proteins are concerned. Investigation of high molecular weight proteins, such as immunoglobulins, becomes a much more difficult problem however. The pioneering research of Porter (1959) on the enzymic digestion of IgG into well-defined smaller fragments eventually made possible the elucidation of the full structure, with its biological implications, of this immunoglobulin of molecular weight $\sim 150,000$ (Edelman et al., 1969).

For a while, investigations on the even larger IgM (molecular weight $8 \times 10^5 - 1 \times 10^6$) were held up, because the proven methods for the fragmentation of IgG did not give satisfactory results when applied to IgM. It was therefore a timeous discovery by Plaut and Tomasi (1970) that trypsin digestion of IgM at high temperatures produced satisfactory yields of smaller fragments, especially the previous elusive pentameric $Fc_5\mu$. The availability of this fragment has greatly facilitated the sequencing of IgM(0u) (Putnam et al., 1972),

while the role of this part of the IgM molecule in biologically important processes, such as placental permeation and complement fixation, could now be investigated. Plaut et al. (1972) have, for example, already determined that $Fc_{5\mu}$ will fix complement and that monomeric Fc_{μ} retains this activity.

This high temperature tryptic digestion was also found extremely useful in the present comparative investigation of normal IgM isolated from Cohn fraction III and the monoclonal IgM from a macroglobulin-aemic patient. This study has provided additional information about the general nature of the trypsin digestion process. In particular the heterogeneity in molecular size of the isolated fragments, even though they appear to be immunogenically pure, has been underlined.

To the author's knowledge, only one literature report (Mihaesco and Seligmann, 1968) refers to the molecular size heterogeneity of Fc_{μ} -fragments produced by enzymic digestion. Their results were obtained on the fragments of monoclonal IgM, obtained by papain digestion in the presence of cysteine. The polydispersity of the Fc_{μ} -fragments isolated by these authors could have been caused, however, by the reducing conditions employed. The $Fc_{5\mu}$ -fragment obtained by Onoue et al., (1968) was also isolated from the papain digest of a monoclonal IgM, but in their investigation, proteolysis was only initiated after removal of free cysteine by Sephadex G-25 desalting of activated papain. Although production of $Fc_{5\mu}$ -fragment was low, a molecular weight of 320,000 and sedimentation coefficient of 10.6 were determined. This is in good agreement with the values reported by Plaut and Tomasi (1970).

In the present investigation the heterogeneity of the tryptic

fragments was readily demonstrated with the aid of SDS-PAGE. This technique introduced by Maizel (1966), is a powerful analytical tool for the determination of size homogeneity and molecular weight (Ahmed-Zadeh et al., 1971). In the field of immunochemistry Parkhouse et al. (1970), have employed SDS-PAGE to follow the reduction and reoxidation of mouse IgM and Zikan and Bennett (1971) investigated the oxidative sulphitolysis of human IgM. However, the usefulness of this technique as a test for the homogeneity of the proteolytic fragments of IgM, has not previously been exploited.

Although the Fab μ -fragments from normal IgM and IgM(Sad) were obtained in high purity, their Fc $_5\mu$ -fragments were distressingly heterogeneous when analysed by means of SDS-PAGE. The purported homogeneity of IgM fragments produced by pepsin, papain and trypsin digestion (see review by Metzger, 1970) may therefore be questionable. The fact that criteria of purity, like IE, Ouchterlony double diffusion, column chromatography and sedimentation analyses, were satisfied (Metzger, 1970), is, in the author's experience, a necessary but not a sufficient basis for assuming molecular size homogeneity.

The cause of the heterogeneity of both the normal IgM and IgM(Sad) Fc $_5\mu$ -fragments remains to be explained. One possibility is to postulate the existence of more than one trypsin-sensitive arginine and lysine bond in the amino acid sequence of the hinge regions of the μ -chains of IgM. Such a sequence has been determined for IgM(Ou) by Paul et al., (1971) and revealed the presence of two arginine and two lysine residues in this region (Fig.13). These authors have established that trypsin cleavage at 60°C and 37°C occurs at arginine 214 and 239 respectively. No scission was obtained at lysine residues 254 or 257. Since it is reasonable to assume that trypsin could either cleave at

both arginine 214 and 239 or at one site only (214) at high temperatures, and since $Fc_5\mu$ consists of five linked $Fc\mu$ -fragments, individual IgM molecules could be cleaved differently to give rise to a pool of $Fc_5\mu$ -fragments with molecular weights differing by as much as: 108 (mean residue weight) $\times 25$ (amino acid residues) $\times 10$ (hinge regions) = $\pm 27,000$ daltons.

An alternative explanation for the molecular heterogeneity of normal $Fc_5\mu$ and $Fc_5\mu$ (Sad) may be that the different bands detected by SDS-PAGE represent not only $Fc_5\mu$, but also lower oligomers with the general formula $Fc(\mu)_n$, where $n < 5$. It is not clear how these lower oligomers would arise, but evidence in support of such a phenomenon can be derived from the finding that after reduction and alkylation of a heterogeneous $Fc_5\mu$ preparation, only a single band of $Fc\mu$ was observed by SDS-PAGE (Chapter 4, Plates 32 and 35). In this regard it is of interest that Putnam et al. (1972) mention that the $Fc_5\mu$ isolated from IgM(0u) was chemically homogeneous and therefore susceptible to sequencing, but that the $Fab\mu$ -fragment was heterogeneous. The existence of μ -chain subclasses (Franklin and Frangione, 1967; Idem, 1968; Mackenzie et al. 1969) may in the case of normal IgM, contribute further to the heterogeneity of its proteolytic fragments.

The suppressive effect of NaCl on the high temperature tryptic digestion of IgM appears to be a novel finding. Attention was first drawn to this effect when IgM(Sad) eluted from a molecular exclusion column (0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0) was not dialysed against 0.05 M-Tris-0.01 M- $CaCl_2$ buffer, pH 8.0 (Plaut and Tomasi, 1970) prior to tryptic digestion. The only tentative explanation that could be offered is that the presence of NaCl induced a conformational change in IgM, which in turn resulted in protection of trypsin sensitive

cleavage points. From an ORD study on the ability of various neutral salts to increase the midpoint of the thermal transition temperature (T_m) of ribonuclease, von Hippel and Wong (1969) concluded that neutral salts induce "high-salt" chain-chain interactions other than those characteristic for the native molecule. If this postulate is extrapolated to the present observations on IgM susceptibility to trypsin fragmentation, it may well be that IgM assumes a "high-salt" conformation of greater stability in the presence of NaCl.

This chapter describes the tryptic fragmentation of normal IgM and IgM(Sad) and the isolation of their respective $Fc_5\mu$ and $Fab\mu$ -fragments. Of these, both normal and monoclonal $Fab\mu$ -fragments were, although immunogenically pure, still molecularly heterogeneous. An investigation on the yields of various fractions obtained by column chromatographic separation of the tryptic digests of IgM(Sad), in the presence of different concentrations of NaCl, clearly showed the inhibitory effect of NaCl on tryptic fragmentation. The origin and the molecular heterogeneity of the $Fc_5\mu$ -fragments is discussed on the basis of the available amino acid sequence analysis of μ -chain. These normal IgM and IgM(Sad) fragments were used for reduction and alkylation studies (Chapter 4) and for physico-chemical analysis (Chapter 5).

Chapter 4

THE PREPARATION OF THE REDUCED AND ALKYLATED COMPONENTS OF INTACT AND TRYPSIN-DIGESTED NORMAL AND MONOCLONAL IgM

4.1 INTRODUCTION

IgM is a pentameric molecule consisting of five IgG-like monomers, called IgM-subunits (IgM_5). These subunits (and therefore the intact molecule) contain a large number of cystine residues (approximately $34/\text{IgM}_5$), which are intimately involved in maintaining the quaternary and tertiary structure of IgM. Disulphide bridges link the five IgM-subunits together circularly and mild reduction of these bonds will invariably lead to depolymerization of IgM. Stronger reducing conditions, especially under dissociating conditions, will also break the inter- μ -L-chain, intra- μ and intra-L-chain disulphide bonds and will lead to complete randomization of the constituent polypeptide chains of IgM. It is also because of the cross-linking of μ - and L-chains by disulphide bridges, that fragments like $\text{F(ab)}_2\mu$ and $\text{Fc}_5\mu$ may be obtained on proteolytic fragmentation of IgM.

Since the first reduction and alkylation studies on IgM by Deutsch and Morton (1957), many detailed investigations on the number of cystine residues and their location in the μ - and L-chains have been undertaken (Miller and Metzger, 1965b, 1966; Suzuki and Deutsch, 1967; Beale and Feinstein, 1969; Beale and Buttress, 1969; Mihaesco and Mihaesco, 1968).

Putnam et al., (1972), have published the detailed primary structure of the μ -chain of IgM(0u) (Fig. 22). It may be seen that the most N-terminal of those cystine residues involved in inter-chain disulphide

bonds link the μ - and L-chains. Proceeding towards the carboxy-terminal end, the next of these is responsible for intrasubunit, inter- μ -chain linkage and is also responsible for the integrity of $F(ab)_2\mu$ proteolytic fragments. Then follows an intersubunit, inter- μ -chain disulphide bond and the one occurring in the penultimate amino acid location is again involved in intrasubunit, inter- μ -chain linkage.

Inspection of a planar model of IgM like that of Dorrington and Mihaesco (1970), together with the knowledge of the position of the disulphide bridges linking the IgG-type subunits, suggests considerable strain on these covalent bonds (Fig. 7). Such a conclusion is contra-indicated however, by the observed highly specific reaggregation of subunits during reoxidation of reduced IgM and the resultant low levels of molecules comprised of more or less than five subunits (Parkhouse et al., 1970).

The important role of cystine residues in the assembly of pentameric IgM has also been shown in an interesting study by Askonas and Parkhouse (1971). They found that newly biosynthesized, intracellular IgM exists in 7-S form and that no spontaneous polymerization to the 19-S species occurred unless it was first subjected to reducing conditions. This suggests the existence of protected thiol groups in the monomer and it was postulated that the removal of such blocking groups takes place shortly before or during secretion of intracellular IgM.

Although plasma IgM occurs predominantly as a pentameric macroglobulin in man, the presence of a natural 7-S form of this molecule has been detected in certain states of disease (Rothfield et al., 1965; Stobo and Tomasi, 1967; Bush et al., 1969). Experimental evidence that this form of IgM is structurally different from IgM₅, has

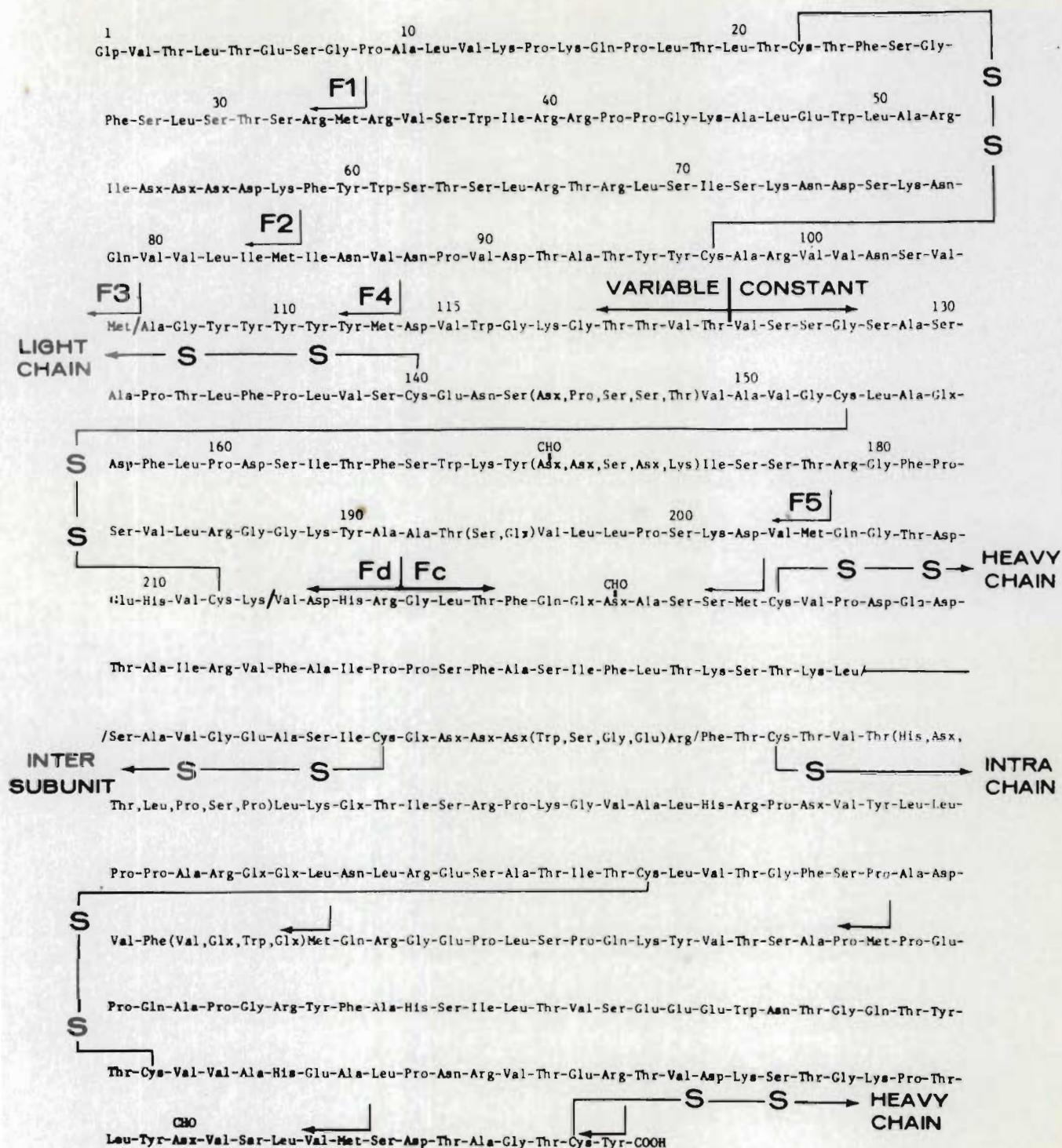


Fig. 22: Amino acid sequence of the μ -chain of IgM(0u) (Putman et al, 1972). The BrCN fragments are denoted F₁, F₂, etc. (taken from Putman et al, 1972).

been found by Dolder, (1971) and Hansson and Nilsson (1972). Dolder has shown that normally occurring 7-S IgM has only one cystine residue available for μ - μ interchain bonding in contrast to three in the case of normal pentameric IgM (Metzger, 1970). The natural occurrence of 7-S IgM in sharks has also been well documented. This form of IgM accounts for 60% of the shark's total immunoglobulin concentration, with the remaining 40% consisting of 19-S IgM (Small et al., 1970). These two forms of IgM are not interconvertible (in vivo) and the 7-S form is thought to play the role of the IgG class of antibody found in higher animals. The question, of course, arises why IgG evolved when IgM in 7-S form can fulfill the same function equally well.

An anomaly in IgM nomenclature should be clarified at this stage. Reference to the polymeric Fc-fragment of IgM as pentameric $F_{C\mu}$ (Plaut and Tomasi, 1970) may cause confusion as this fragment consists of ten covalently linked carboxy-terminal μ -chain fragments. It should therefore rather be called decameric $F_{C\mu}$ (see for instance Putnam et al., 1972). By inference the reductive subunits of $F_{C_5\mu}$ should be called monomeric $F_{C\mu}$ where in fact it consists of two equal $F_{C\mu}$ peptides. The author will refer to polymeric $F_{C\mu}$ as $F_{C_5\mu}$ (Plaut and Tomasi, 1970) and to the subunits obtained after reduction and alkylation as $F_{C\mu}$ (non-dissociating conditions) and monomeric $F_{C\mu}$ (dissociating conditions).

This chapter describes the purification of IgM subunits (IgM_S), μ -chain, L-chain and monomeric $F_{C\mu}$ from both normal IgM and IgM(Sad). All these compounds were obtained by reduction and alkylation of the appropriate precursors.

4.2 MATERIALS

Normal IgM, IgM(Sad) and their respective $F_{C_5\mu}$ and $F_{ab\mu}$ fragments were isolated as described in chapters 2 and 3.

Dithiothreitol (DTT) was a product of Seigaku Fine Biochemicals, Tokyo, Japan, and iodoacetamide (IA) was purchased from Sigma Chemical Company, Missouri, U.S.A.

4.3 METHODS

4.3.1 Immunological methods

Ouchterlony double diffusion and immunoelectrophoresis were carried out as previously described (Chapter 2).

4.3.2 Analytical ultracentrifugation

Sedimentation was done on a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, California, U.S.A.) equipped with a schlieren light source, electronic speed control and automatic rotor temperature indicator and control system (RTIC). Protein samples at approximately 1% (w/v) concentration dissolved in 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0, were centrifuged (20°C) in a single sector aluminium centre-piece using sapphire windows and the An-D rotor.

4.3.3 Quantitation of bands separated by SDS-PAGE

The composition in terms of μ - and L-chain of reduced and alkylated IgM(Sad) was determined by scanning of the polyacrylamide gel cylinder shown in Plate 37b (Chromoscan, Joyce-Loebl, Gateshead, England). The percentage composition of the two chains obtained after reduction and alkylation of normal Fab μ (Plate 41c) was determined similarly. Quantitation of the areas under peaks was effected by allowing the instrument to integrate these curves automatically on a repeat scan.

4.3.4 Reduction and alkylation of normal IgM, IgM(Sad) and their respective tryptic fragments

Samples for chromatography or SDS-PAGE analysis were dissolved in 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0, and reduced for 1 h at 37°C in the presence of 0.01 M-DTT. After rapidly cooling the reaction mixture to 4°C, reduction was stopped by alkylation of thiol groups. This was done by addition of crystalline iodoacetamide to a 10% molar excess over available reducing agent (thiol groups) and maintaining the pH at 8.0 for 0.5 h. The same procedures were applied to samples dissolved in dissociating media (0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0).

4.3.5 Purification of reduced and alkylated components by molecular exclusion chromatography

Reduced and alkylated normal Fc_{5u} and $Fc_{5u}(Sad)$ were purified by chromatography on Sephadex G-100 (superfine) with 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0.

Monomeric Fc_u as well as the H- and L-chains of normal IgM and IgM(Sad) were isolated on Sephadex G-200 columns in the dissociating medium 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0.

The reduced and alkylated 7-S components of normal IgM and IgM(Sad) were also purified on Sephadex G-200, but the eluting buffer (0.05 M-Tris-0.5 M-NaCl, pH 8.0) did not contain any GuHCl.

4.4 RESULTS

4.4.1 Purification of normal Fc_u

Chromatography of reduced and alkylated normal Fc_{5u}

yielded a single major peak (Fig.23, fraction a) which was found to be almost pure F_{cu} by SDS-PAGE analysis (Plate 31a). The SDS-PAGE analysis of fraction 23b (Plate 31b) yielded the rather perplexing result that this fraction, although eluting as a smaller molecular weight molecule than fraction 23a, had exactly the same electrophoretic mobility as the latter under the dissociating conditions of SDS-PAGE.

Further purification of normal F_{cu} was achieved by chromatography on Sephadex G-200 in the presence of $GuHCl$ (Fig. 24). The SDS-PAGE analysis of the rather broad fraction 24a showed that it consisted of a mixture of components of higher molecular weight than the pure normal F_{cu} , obtained in fraction 24b (Plate 32). IE analysis (Plate 33) of purified normal F_{cu} and its pentameric precursor, F_{c5u} , indicated little electrophoretic difference between these two proteins, but a difference in diffusion coefficients was obvious from their positions relative to the antiserum trough.

4.4.2 Purification of $F_{cu}(Sad)$

Unlike reduced and alkylated normal F_{c5u} , the chromatogram obtained for reduced and alkylated $F_{c5u}(Sad)$ (Fig. 25) showed the presence of two major peaks. SDS-PAGE analysis of fractions 25a, 25b and 25c (Plate 34) however showed the second fraction (25b) to contain the major $F_{cu}(Sad)$ component and IE analysis of fraction 25b as well as of its precursors before and after reduction and alkylation is shown in Plate 35. Fractions 25a and 25c produced no precipitin lines and are not shown in Plate 35. From these analyses,

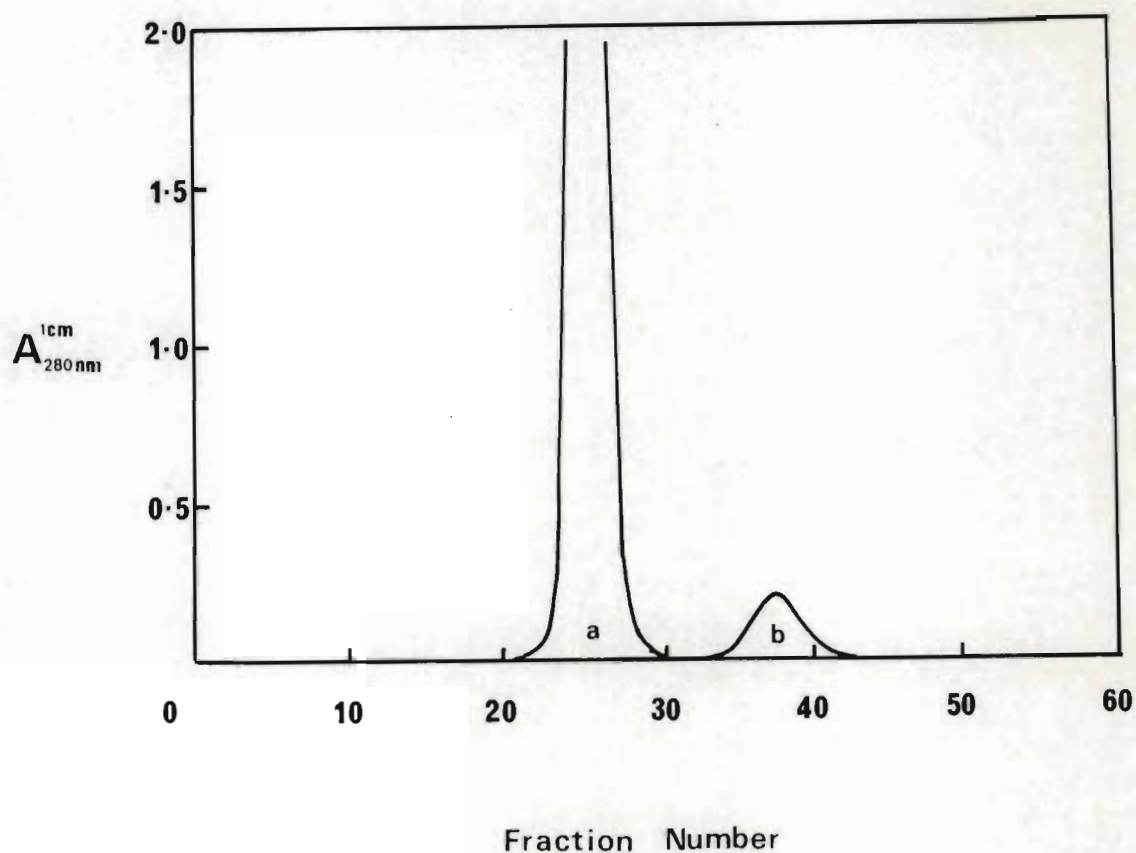


Fig. 23: Elution pattern after chromatography of reduced and alkylated normal $Fc_{5\mu}$ (30 mg fraction 16b) on Sephadex G-100 superfine (column, 2 x 85 cm; buffer, 0.05 M-Tris-0.5 M-NaCl, pH 8.0; flowrate 15 ml/h; 5 ml fractions).

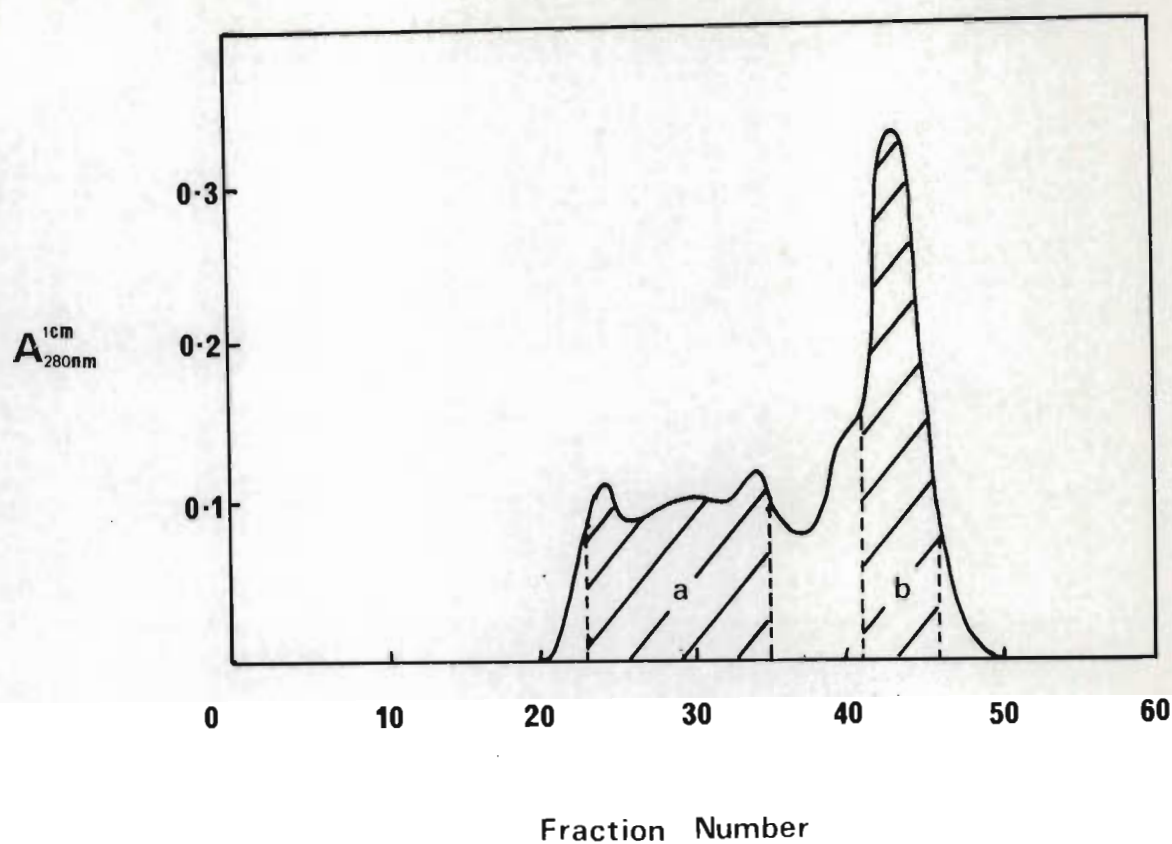


Fig. 24: Elution pattern after rechromatography of reduced and alkylated normal Fc μ (21 mg of fraction 23a) on Sephadex G-200 (column, 2.5 x 90 cm; buffer, 0.01 M-Tris-4.0 M-GuHCl, pH 8.0; flowrate 7.5 ml/h; 5 ml fractions).

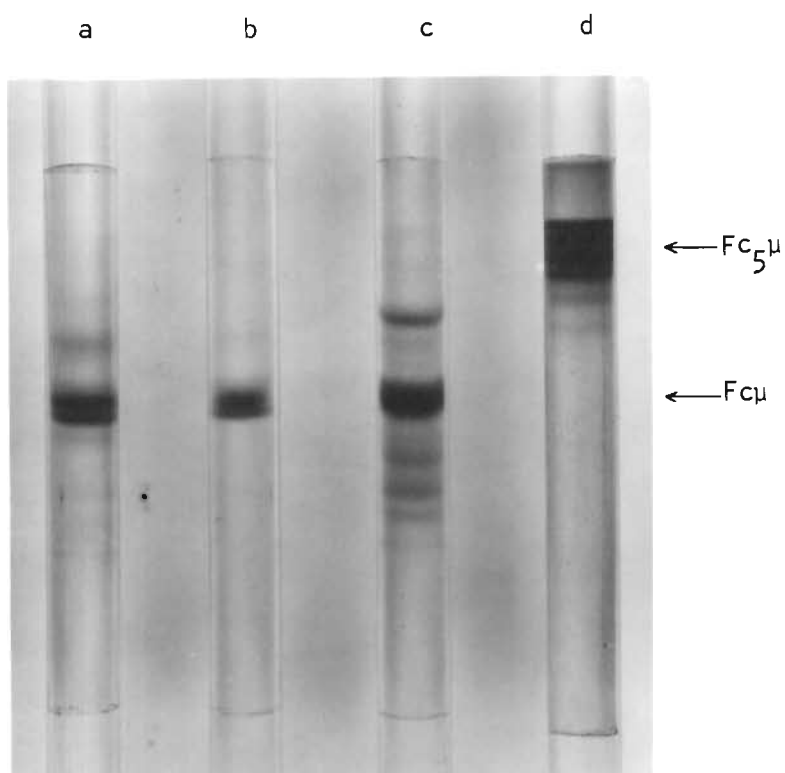


Plate 31 SDS-PAGE (5.6% gel) analysis of (from left to right) fractions 23a, 23b, reduced and alkylated normal $Fc_{5\mu}$ and normal $Fc_{5\mu}$ (4.0% gel).

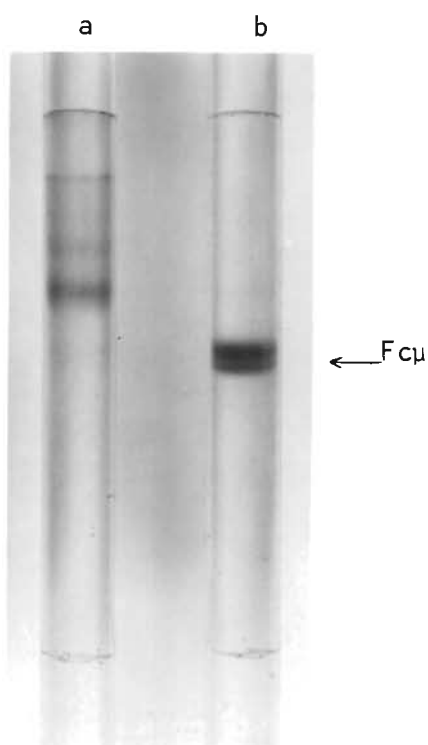


Plate 32 SDS-PAGE (5.6% gel) analysis of fractions 24a and 24b.

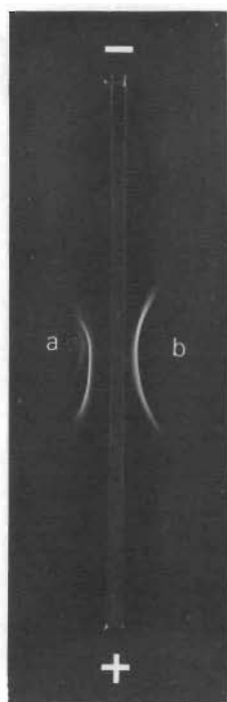


Plate 33 IE analysis of (a) normal Fc_5^u and (b) fraction 24b against unadsorbed anti-IgM antiserum.

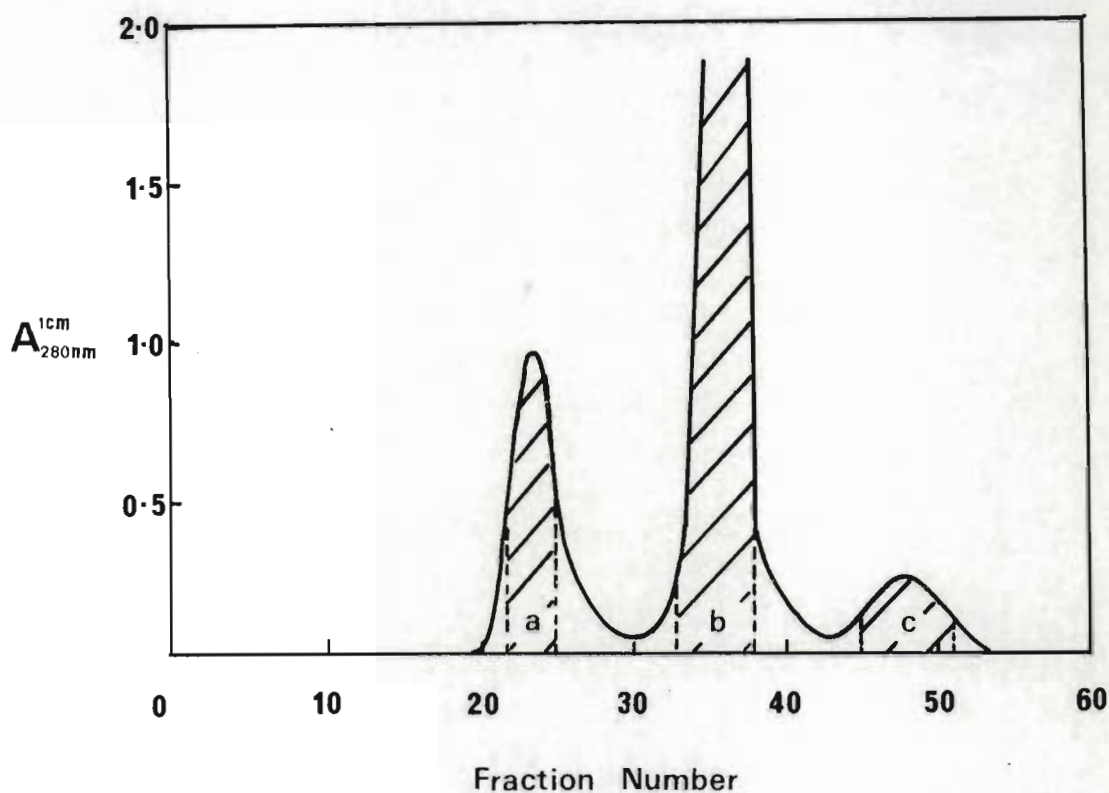


Fig. 25: Elution pattern after chromatography of reduced and alkylated $Fc_{5\mu}(Sad)$ (42 mg fraction 20b) on Sephadex G-100 superfine (column, 2.5 x 90 cm; buffer, 0.05 M-Tris-0.5 M-NaCl, pH 8.0; flowrate 15 ml/h; 5 ml fractions).

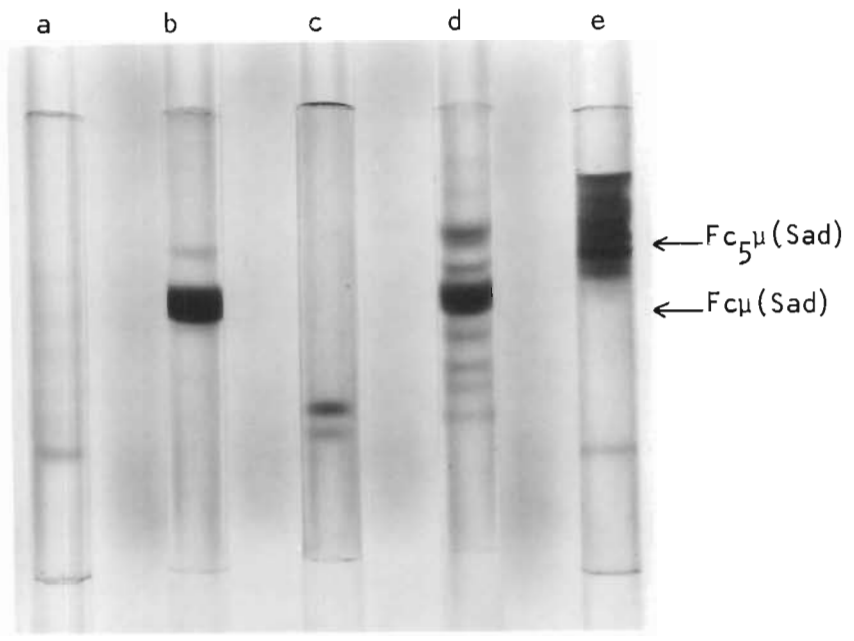


Plate 34 SDS-PAGE (5.6% gel) analysis of (from left to right) fractions 25a, 25b, 25c and $Fc_{5\mu}(Sad)$ after and before reduction and alkylation (4.0% gel).



Plate 35 IE analysis of (from left to right) $Fc_{5\mu}(Sad)$ before (a) and after (b) reduction and alkylation and of fraction 25b against unadsorbed anti-IgM antiserum.

fraction 25b could be identified as monomeric $F_{\mu}(Sad)$ and because of its good quality (Plate 34b), no further purification was necessary.

4.4.3 Preparation of reduced and alkylated heavy and light-chains of normal IgM

Purified normal IgM dissolved in GuHCl buffer to bring about the complete unfolding of its peptide chains and the concomitant exposure of all cystine bonds gave, after reduction and alkylation, the separation of μ - and L-chains shown in Fig. 26. SDS-PAGE analysis showed that the mobilities of fractions 26a and 26b are compatible with those expected for the component μ - and L-chains of IgM (Plate 36).

4.4.4 Preparation of heavy and light-chains of IgM(Sad)

Reduction and alkylation of IgM(Sad) and separation of its constituent μ - and λ -chains was achieved as described above (section 4.4.3) and yielded the chromatogram shown in Fig. 27. Plate 37 depicts the SDS-PAGE analysis of fractions 27a and 27b and of reduced and alkylated IgM(Sad).

Rechromatography of fractions 26a and 27a on the same column yielded highly purified μ -chain preparations. (Plate 38).

4.4.5 Purification of normal IgM_s and IgM_s(Sad)

The Sephadex G-200 chromatograms of normal IgM and IgM(Sad) reduced and alkylated under non-dissociating conditions are shown in Fig. 28. The nature of the minor peaks shown in Fig. 28 is unknown, but they yielded poor and indistinct precipitin lines when analysed by IE against

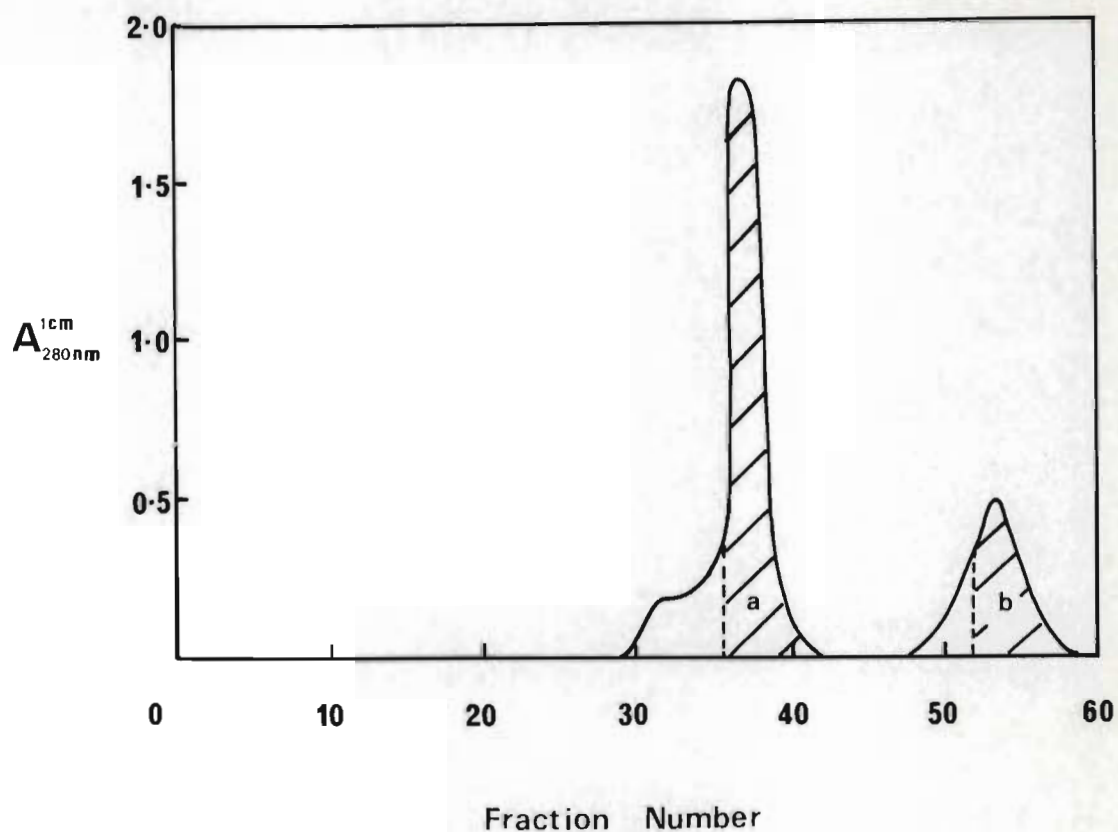


Fig. 26: Elution pattern after chromatography of reduced and alkylated normal IgM (61 mg) on Sephadex G-200 (column, 2.5 x 92 cm; buffer, 0.01 M-Tris-4.0 M-GuHCl, pH 8.0; flow-rate, 7.5 ml/h; 5 ml fractions).

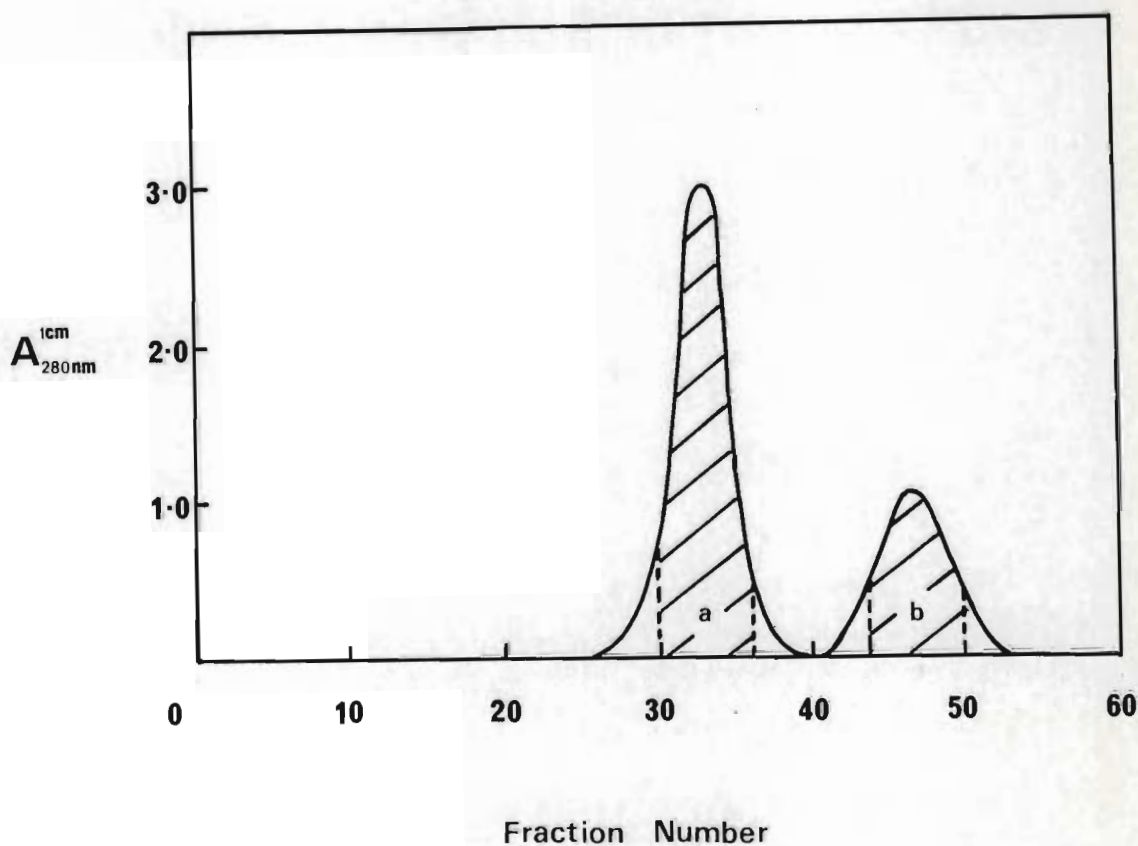


Fig. 27: Elution pattern after chromatography of reduced and alkylated IgM(Sad) (100 mg) on Sephadex G-200 (column, 2.5 x 92 cm; buffer, 0.01 M-Tris-4.0 M-GuHCl, pH 8.0; flowrate, 7.5 ml/h; 5 ml fractions).

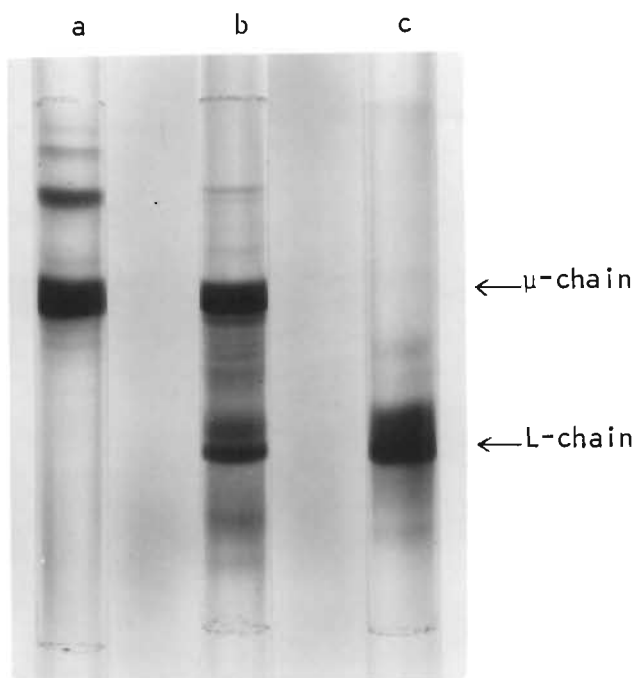


Plate 36 SDS-PAGE (5.6% gel) analysis of (a) fraction 26a, (b) reduced and alkylated normal IgM, (c) fraction 26b.

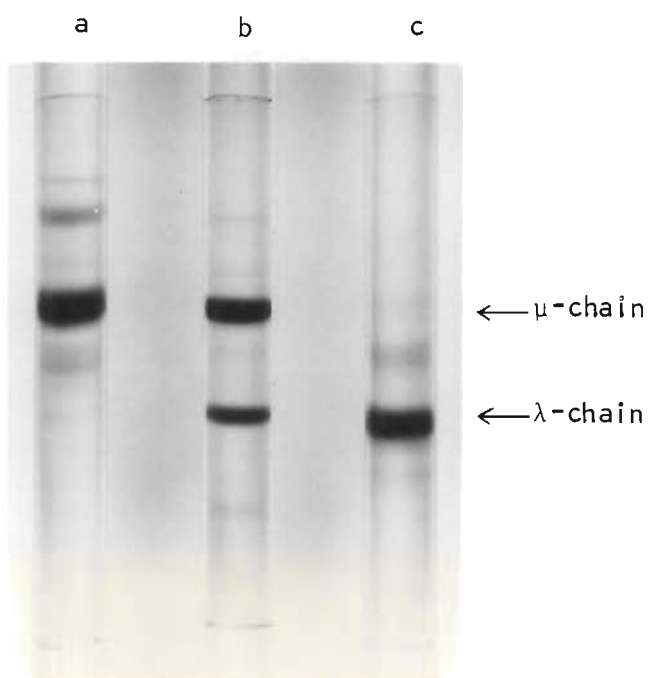


Plate 37 SDS-PAGE (5.6% gel) analysis of (a) fraction 27a, (b) reduced and alkylated IgM(Sad), (c) fraction 27b.

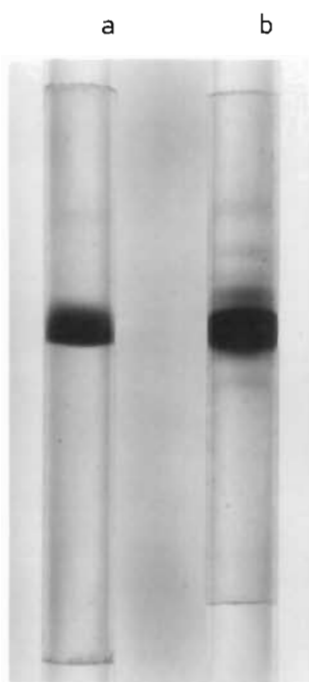


Plate 38 SDS-PAGE (5.6% gel) analysis of
(a) rechromatographed fraction 26a and (b)
rechromatographed fraction 27a.

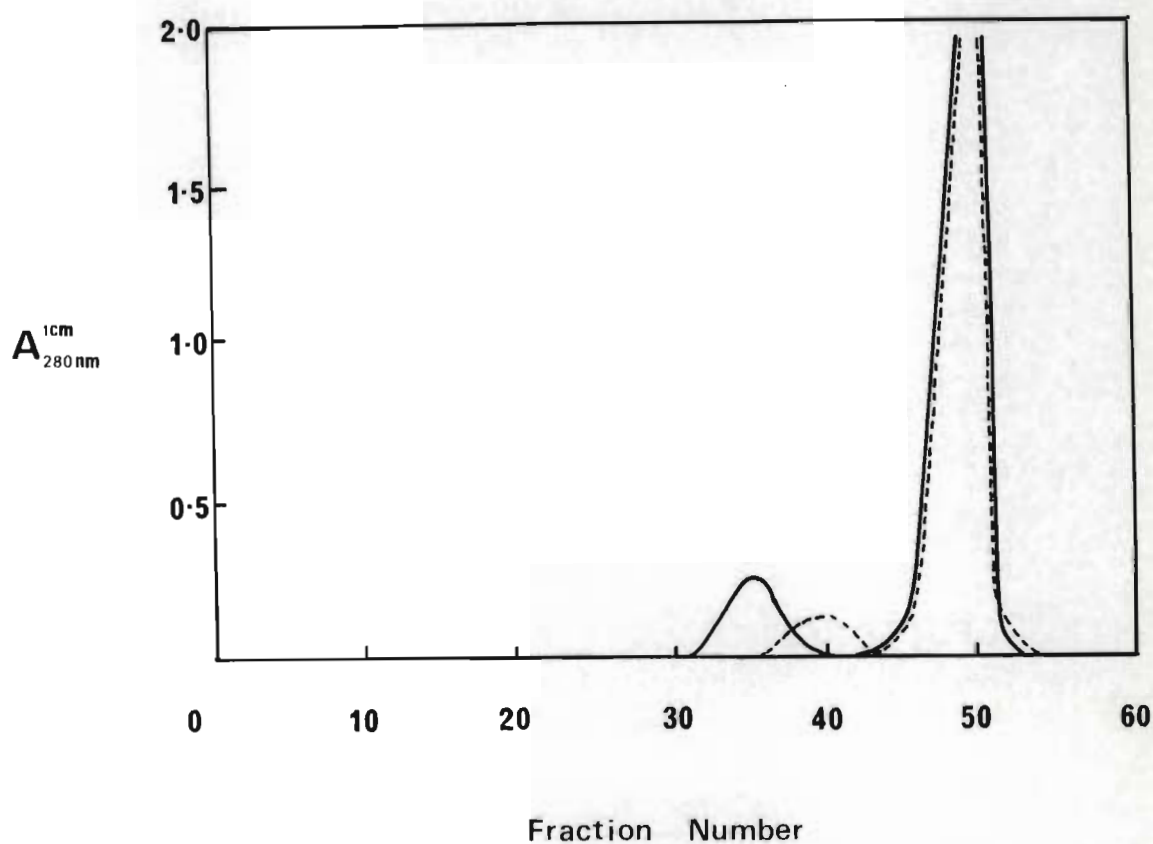


Fig. 28: Elution pattern after chromatography of reduced and alkylated normal IgM (100 mg, —) and reduced and alkylated IgM(Sad) (100 mg,) on Sephadex G-200 (column, 2.5×92 cm; buffer, 0.05 M-Tris-0.5 M-NaCl, pH 8.0; flow-rate, 10 ml/h; 5 ml fractions).



Plate 39 Sedimentation analysis of (A) reduced and alkylated IgM(Sad) and (B) reduced and alkylated normal IgM. Photographs were taken 30 min (A) and 24 min (B) after reaching 56,000 r.p.m. Sedimentation is from left to right.

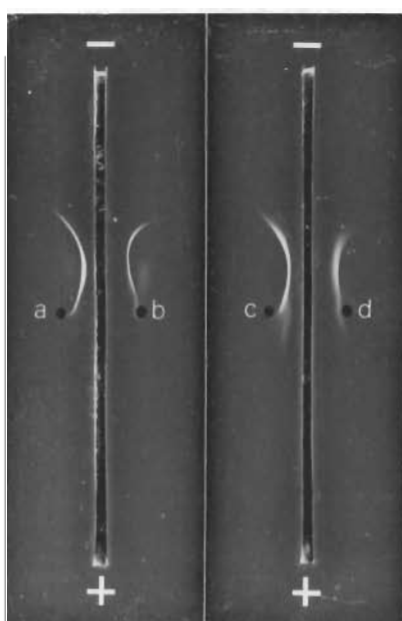


Plate 40 IE analysis of (a) IgM_s(Sad), (b) IgM(Sad), (c) normal IgM_s and (d) normal IgM against unadsorbed anti-IgM antiserum.

anti-IgM antiserum (not shown). Sedimentation analysis of the respective 7-S subunits (IgM_S) showed asymmetrical sedimentation peaks for both these components (Plate 39). IE comparison of normal IgM_S and $\text{IgM}_S(\text{Sad})$ and their respective 19-S precursors is shown in Plate 40 and clearly illustrates the higher diffusion coefficients of the smaller IgM species.

4.4.6 SDS-PAGE analysis of reduced and alkylated normal Fab_μ and $\text{Fab}_\mu(\text{Sad})$

The SDS-PAGE analysis of reduced and alkylated normal Fab_μ , $\text{Fab}_\mu(\text{Sad})$ and λ -chain isolated from $\text{IgM}(\text{Sad})$ is shown in Plate 41. The interesting observation that reduced and alkylated normal Fab_μ yielded double bands (Plate 41c) is discussed later.

4.4.7 Quantitation of bands resolved by SDS-PAGE

The high background caused by multiple minor bands in the SDS-PAGE analysis shown in Plate 36b, made it impossible to estimate the μ - and L-chain composition of normal IgM by densitometric scans of this gel. Scanning of the SDS-PAGE gels of reduced and alkylated $\text{IgM}(\text{Sad})$ (Plate 37b) yielded the results shown in Table 2.

Quantitation in similar manner of the two bands obtained on reduction and alkylation of normal Fab_μ (Plate 41c) yielded percentages of 69 and 31 for the upper and lower bands respectively.

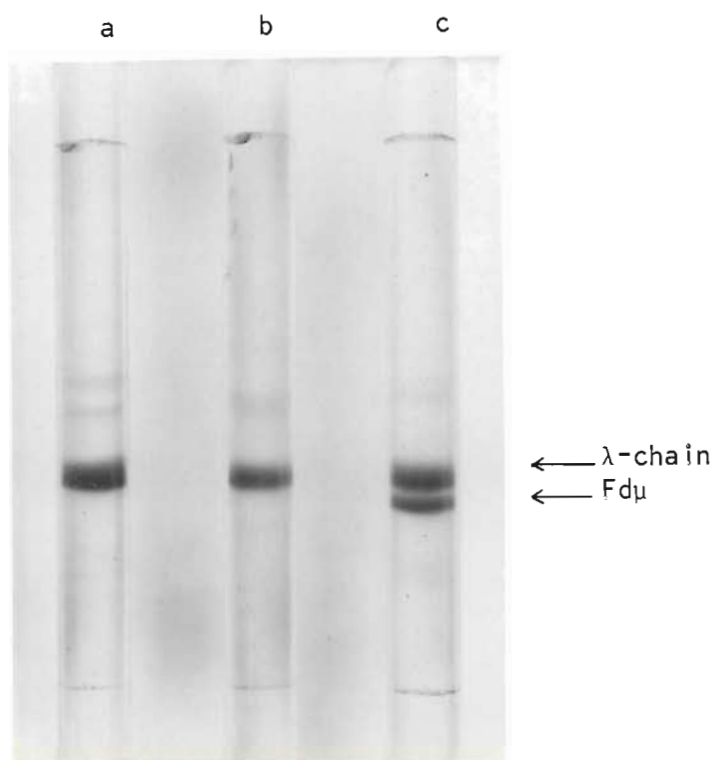


Plate 41 SDS-PAGE (5.6% gel) analysis of (a) reduced and alkylated Fab μ (Sad) (fraction 21a), (b) λ -chain and (c) reduced and alkylated normal Fab μ (fraction 17a).

Table 2 The μ - and λ -chain composition of IgM(Sad) calculated from densitometric scans of SDS-PAGE gels of a reduced and alkylated preparation:

Chain	Observed %	Theoretical % composition (weight basis ^a)	
		$10\mu + 10\lambda$ ^b	$10\mu + 15\lambda$ ^c
μ -chain	68	72.5	63.7
λ -chain	32	27.5	36.3

a: Calculated from the molecular weight values determined for IgM(Sad) μ - and λ -chain (Chapter 5).

b: $10\mu + 10\lambda$ -chain model proposed by Miller and Metzger (1965b).

c: $10\mu + 15\lambda$ -chain model proposed by Suzuki and Deutsch (1967).

4.5 DISCUSSION

The fact that normal $Fc_5\mu$ yielded a single major component after reduction and alkylation (Plate 31c) could possibly indicate that the multiple bands shown by SDS-PAGE of the parent trypsin fragment (Plate 31d) represented Fc_μ oligomers such as $Fc_5\mu$, $Fc_4\mu$, $Fc_3\mu$, etc. It is not clear how such oligomers could arise from the intact pentameric IgM molecule under non-reducing digestion conditions, unless trypsin cleavage sites exist on either side of the cystine residues involved in intersubunit linkage. Inspection of the amino acid sequence of the μ -chain of IgM(0u) (Putnam *et al.*, 1972) reveals that this is feasible. In the case of this μ -chain, trypsin cleavage occurs 8 and 9 residues removed on either side of the specific cystine residue (position not yet accurately determined) involved in intersubunit linkage (Fig. 22). A loss of two or more of these octadeca-peptides containing the cystine residue vital for the pentameric $Fc_5\mu$ structure will lead to Fc_μ fragments of

lower molecular weight.

The almost identical SDS-PAGE patterns of chromatographic fractions 23a and 23b indicated that some degree of dissociation of F_{μ} , which normally exists as a dimer under non-dissociating conditions, occurred during column chromatography. The presence of vague double bands observed after gel chromatography and SDS-PAGE analysis of monomeric normal F_{μ} (Plate 31a) was reminiscent of similar doublets given by normal $F_{ab\mu}$ after ion exchange chromatography (Plate 20). Minor components were successfully removed from normal monomeric F_{μ} by re-chromatography under dissociating conditions (Plates 32 and 33).

The molecular heterogeneity of $F_{C_5\mu}(\text{Sad})$ (Plate 34e) was similar to that observed for normal $F_{C_5\mu}$. The postulate of trypsin "scooping out" that area of the μ -chain containing the intersubunit disulphide bridge, is perhaps even more applicable in the case of $\text{IgM}(\text{Sad})$, since a small peptide, fraction 19c, could be isolated from the digest (cf. Chapter 3). This peptide was identified as a subfragment of $F_{\mu}(\text{Sad})$, most probably constituting the N-terminal portion of the molecule (Chapter 3). The observation of similar small fragments after reduction and alkylation of $F_{C_5\mu}(\text{Sad})$ (Plate 34a and 34c) is also in agreement with such an explanation.

The homogeneity of monomeric $F_{\mu}(\text{Sad})$ after one chromatography run (Plate 34b) made further purification unnecessary.

Separation of the μ - and L-chains of normal IgM and $\text{IgM}(\text{Sad})$ was achieved in the presence of 4 M-GuHCl (Lamm et al., 1966). The choice of this dissociating agent, rather than the more popular propionic or acetic acids, was decided on, because of the poor chromatographic resolution given by the latter two for γ - and L-chain.

The large number of minor bands, in addition to the two major μ - and L-chain bands, shown by SDS-PAGE analysis of reduced and alkylated normal IgM (Plate 36b), is in sharp contrast to the clear resolution into μ - and L-chain only, in the case of reduced and alkylated IgM(Sad) (Plate 37b). The presence of the minor components in the case of normal IgM could perhaps be ascribed to limited proteolysis of normal IgM by the protease plasmin, which might have occurred during the isolation procedure. This enzyme is known to be concentrated in Cohn fraction III (Sgouris et al., 1960). Rechromatography of the respective μ -chains on the same media, yielded material of high purity (Plate 38).

When non-dissociating solvents were employed during the reduction and alkylation of normal IgM and IgM(Sad) to obtain their respective monomers, chromatography yielded two peaks in each case (Fig. 28). No precipitin lines were obtained on IE analysis of the material in either of the two minor components eluted first. The only difference seen in the IE patterns of the two major fractions (normal IgM_S and IgM_S(Sad)) and their respective precursors was that the curvature and relative distance of the precipitin lines from the antiserum troughs varied (Plate 40). Sedimentation analysis (Plate 39) of these 7-S monomers showed a larger trailing edge for normal IgM_S than for IgM_S(Sad). The nature of the slower sedimenting material shown in Plate 39 is unknown, but could be due to L-chain (Suzuki and Deutsch, 1967).

The reduction and alkylation products of Fab μ (Sad) are λ -chain and Fd μ and could not be separated by SDS-PAGE because of their very similar molecular weights (Plate 41a). The molecular weights of intact Fab μ (Sad) was determined to be 47,300 daltons (Chapter 5) and that of λ -chain is known to be 23,400 daltons (Putnam, 1967). The difference between these two molecular weights, 23,900 daltons, is that of the Fd μ -piece.

The SDS-PAGE analysis of reduced and alkylated normal Fab μ (Plate 41c), however, showed two bands. The uppermost of these could be identified as L-chain by comparison of its electrophoretic mobility with that of purified λ -chain (Plate 41b). The Fd μ -piece of normal Fab μ , or perhaps only part thereof, therefore appeared to be of smaller molecular weight than its accompanying L-chain. Scanning of the polyacrylamide gel cylinder shown in Plate 41c, yielded a ratio of 69 : 31 for the upper and lower bands respectively. This could only mean that the upper band consisted of both L-chain and an Fd μ -piece of bigger molecular weight than that found in the lower band. This observation may also explain the occurrence of double bands found in the SDS-PAGE analysis of normal Fab μ shown in Plate 20.

Metzger (1970) states that he has never observed values higher than 27% for the L-chain content of IgM. The author's value of 32% for IgM(Sad) (Table 2) is therefore rather high. This value is however the same as that found by Suzuki and Deutsch (1967) who originally proposed a 10 μ + 15L-chain model for IgM. There is no reason to doubt the accuracy of the percentage yields determined for μ - and λ -chain (Table 2), but factors like the possible differential staining of these two peptides or their different carbohydrate contents may give rise to biased estimates. The percentage composition of λ -chain (32%), determined by scanning, correlates poorly with that calculated for a 10 μ + 15 λ -chain model (36.3%) from molecular weight data (Table 2). Nevertheless, this model was the only one to satisfy the molecular weight of 970,000 daltons determined for IgM(Sad) (Chapter 5).

The main objective of the work reported in this chapter, namely the preparation of H-chains, 7-S reductive subunits and monomeric Fc μ fragments from normal IgM and IgM(Sad), was achieved. While analysis of reduced

and alkylated normal $\text{Fab}\mu$ revealed the presence of distinct double bands in the SDS-PAGE pattern, only a single major band was obtained from reduced and alkylated normal $\text{Fc}_5\mu$ and $\text{Fc}_5\mu(\text{Sad})$ and this finding is discussed on the basis of the amino acid sequence of a monoclonal IgM. Investigation of the μ - and λ -chain composition of IgM(Sad) did not resolve the uncertainty regarding the number of λ chains in this molecule.

Chapter 5

PHYSICO-CHEMICAL CHARACTERISATION OF NORMAL IgM, IgM(Sad) AND THEIR DERIVATIVES

5.1 INTRODUCTION

The structure and conformation of a protein determine to a large extent its biological function, and hence structural and conformational studies are of vital importance. These may be conducted at various levels since proteins may be described with reference to their primary, secondary, tertiary or quaternary structures.

The primary structure entails the amino acid sequence of the polypeptide chains comprising the protein and becomes important in genetic studies and in determining such parameters as the positions of disulphide bonds. However, Metzger, (1970) states that "the amino acid composition of IgM is unremarkable when compared to other immunoglobulins and it is clear from several detailed amino acid compositions (Suzuki and Deutsch, 1967; Putnam et al., 1967) that such data cannot be used to build molecular models - i.e. any number of light and/or heavy chains of any assumed size would give substantially the same composition for IgM." Bearing this in mind, therefore, in this investigation amino acid analysis was used only to detect differences in the amino acid content of related fractions with dissimilar electrophoretic mobilities, or alternatively to obtain evidence regarding the origin of fragments.

Tertiary and quaternary characteristics of proteins are reflected in their physical properties and the ultracentrifuge, described by Bull (1941) in the very first article to appear in *Advances in Enzymology* as "the most important tool ever devised for the physical study of proteins,"

has been used extensively for this purpose in the divergent subdisciplines of Biochemistry.

The ultracentrifuge has played a vital role in the characterisation and elucidation of the structure of the immunoglobulins. Examination of purified IgM in the analytical ultracentrifuge usually reveals three or more well separated sedimentation peaks. The major component sedimenting at 18-19S (although a range of 14.2S to 22.2S has been found) is often preceded by larger IgM species with sedimentation coefficients of 29S, 38S and higher (Deutsch and Morton, 1958; Müller-Eberhard and Kunkel, 1959). Such species with sedimentation coefficients greater than 19S cannot be ascribed to artefacts introduced by the isolation procedure, because these molecules may also be detected in normal and macroglobulinaemic sera. Although it has been shown that these IgM species ($S > 35$) bind complement (C1q) with greater avidity than those with a lower sedimentation coefficient (Augener et al., 1971), it is still not clear whether aggregated IgM has a specialised biological function.

The range of sedimentation coefficients found for the major component of IgM has also been borne out in molecular weight determinations. Molecular weight values of 620,000 to 1,180,000 daltons have been reported for different Waldenström IgM's (Fillitti-Wurmser and Hartmann, 1968), but it is today accepted that 850,000-900,000 daltons is a more realistic range (Metzger, 1970).

Ultracentrifugal studies also led to the present model for IgM. Following the early observation by Deutsch and Morton (1957) on the dissociating effect of reduction of IgM, several investigators prepared subunits in which the constituent polypeptide chains remained linked by disulphide bridges (Miller and Metzger, 1965b; Morris and Inman, 1968) and

molecular weight determinations suggested that the 19S IgM species consisted of 5 of these subunits which are covalently linked.

Proteolytic fragments were also investigated and confirmed the proposed structure for IgM. Metzger (1970) has reported a variation in the molecular weights for tryptic $\text{Fab}\mu$ and $\text{F(ab)}_2\mu$ fragments, possibly due to the variability in the point of μ -chain scission, but average values of 45,000 daltons for $\text{Fab}\mu$ and 118,000 daltons for $\text{F(ab)}_2\mu$ may be accepted. Plaut and Tomasi (1970) purified $\text{Fc}_5\mu$ -fragments from high temperature trypsin digests and found molecular weight values of 340,000 daltons for the intact fragment and 67,000 daltons for $\text{Fc}\mu$ fragments obtained after reduction of $\text{Fc}_5\mu$. Calculation of the molecular weight of an intact molecule from data obtained for the proteolytic fragments, supports the notion that 5 subunits are covalently linked to form the intact Waldenström IgM.

The physico-chemical characterisation of immunoglobulins by means of molecular weight determinations, however, yields only limited information about the conformation of these molecules and recourse must be had to other techniques if a more complete picture is desired. Thus elegant ultrastructural studies have been undertaken with the electron microscope on normal human and rabbit as well as Waldenström IgM preparations (Svehag *et al.*, 1967a; 1967b; Chesebro *et al.*, 1968) and on a mouse myeloma IgM (Parkhouse *et al.*, 1970). These electron micrographs showed that the diameter of the intact IgM molecule is approximately 330-350 Å and that of the pentameric $\text{Fc}_5\mu$ -fragment about 105 Å (Parkhouse *et al.*, 1970). In the detailed electron micrographs of Parkhouse *et al.* (1970), the dimeric ultrastructure of the F(ab)_2 part of mouse IgM is clearly visible and the flexibility of the area where they join (hinge region) is well

illustrated by the observation of "bracket-like" structures when IgM attaches itself to antigen (Feinstein and Munn, 1969).

Although the only absolute technique for conformational analysis is X-ray crystallography, the lack of suitable crystalline material has, until recently, prevented its application to the immunoglobulins or their fragments. Such studies have now, however, been carried out on IgG (Sarma et al., 1971; Edmundson et al., 1970), isolated L-chains (Schiff-er et al., 1969) and on Fab, Fc and V_L fragments (Solomon et al., 1970). The X-ray diffraction pattern of a human myeloma Fab'γ fragment (Poljak et al., 1972) has provided very good evidence in support of the existence of compact domains in IgG, as deduced earlier (Edelman et al., 1969) from amino acid sequence analysis. However, the resolution attained in current X-ray crystallographic studies on the immunoglobulins is still too low to furnish a detailed picture, albeit a static one, of their conformation.

Further valuable information regarding the conformation of the immunoglobulins in solution may be gained, however, from optical rotatory dispersion (ORD) and circular dichroism (CD) spectra. Both CD and ORD depend on the presence of elements of asymmetry in the molecular structure, but CD yields simpler and more accurately interpretable results as the CD ellipticity bands are more discreet and usually appear on a background of zero ellipticity. Furthermore, the influence of chromophores at wavelengths far removed from the wavelength region under study is negligible in the case of CD spectra. In the present study therefore, only the CD spectra of the various IgM entities were determined for comparative purposes.

In this chapter are described some of the physico-chemical character-

istics of normal IgM and IgM(Sad). In order to obtain information from analytical ultracentrifugation experiments, it is preferable to use monodisperse (i.e. homogeneous) protein preparations. The relevant purification procedures applied to the different fragments and peptides of normal IgM and IgM(Sad) have been described in chapters 3 and 4. The molecular weights and sedimentation coefficients of normal IgM, IgM(Sad) and some of these subfragments and polypeptide chains have been determined and compared with each other and those in the literature. Interesting differences between the CD spectra of normal Fab μ and Fab μ (Sad) fragments, as well as IgM(Sad) before and after denaturation by GuHCl were also observed. The small fragment found in trypsin digests of IgM(Sad) (fraction 19c) had a CD spectrum which differed completely from those of the other IgM derivatives and suggested that it exists as a randomly coiled molecule. These results are also compared with those in the literature. It is concluded that the pentameric structure proposed for a Waldenström IgM (Miller and Metzger, 1965b), is also valid for normal IgM isolated from Cohn fraction III.

5.2 MATERIALS

Normal IgM, IgM(Sad) and their tryptic and reduced and alkylated fragments were purified as described in chapters 2, 3 and 4. Prior to molecular weight determinations, normal IgM and IgM(Sad) were rechromatographed in 4M-GuHCl on Sepharose 6B.

Ultrapure GuHCl with an absorbance of less than 0.03 at 280 nm for a 6M solution, was purchased from Swartz-Mann, Orangeburg, New York, U.S.A.

5.3 METHODS

5.3.1 Amino acid analysis

Duplicate samples (2 - 3 mg) of freeze-dried material were hydrolysed in vacuo for 24 h at 110°C with excess 6 N HCl and subsequently prepared for amino acid analysis on a Beckman Model 120 B automatic amino acid analyzer according to the methods of Spackman et al. (1958).

5.3.2 Determination of sedimentation coefficients

Analytical ultracentrifugation was performed on a Beckman Model E ultracentrifuge (Chapter 4). Sedimentation coefficients were determined on protein solutions previously dialysed for 24 h against 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0. A single-sector aluminium centrepiece, sapphire windows and an An-D rotor were routinely used for sedimentation analysis. The schlieren photographs were analysed by means of a Nikon Model 6C Profile Projector (Nippon Kogaku K.K., Tokyo, Japan).

Sedimentation coefficients ($S_{20,w}$) were calculated from the data of at least five different protein concentrations according to the equation:

$$S_{20,w} = S_{\text{obs}} \left(\frac{\eta_t}{\eta_{20}} \right) \left(\frac{\eta_{\text{sol}}}{\eta_w} \right) \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{t,\text{sol}}} \right)$$

where η_t = viscosity of water at temperature (t) of centrifuge run;

η_{20} = viscosity of water at 20°C;

η_{sol} = viscosity of sample solution at known temperature (t');

η_w = viscosity of water at known temperature (t');

$\rho_{20,w}$ = density of water at 20°C;

$\rho_{t,sol}$ = density of sample solution at temperature (t) of centrifuge run;

\bar{v} = partial specific volume.

Sedimentation coefficients at infinite dilution ($S_{20,w}^0$) were determined by regression analysis of a plot of $S_{20,w}$ against concentration (absorbance) units. Both of the above calculation procedures were performed on an IBM 1130 computer with the aid of a special programme; the programme listing together with an example of a data input sheet and the printout for a set of calculated results are given in Appendix 1.

Values for the partial specific volumes were taken from Dorrington and Mihaesco (1970); since no GuHCl was used in the present determination of sedimentation coefficients, the \bar{v} -values were corrected by the addition of 0.01 ml/g to each.

5.3.3. Determination of molecular weights

Proteins were dissolved and extensively dialysed (3 days) in either 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0, or 6.0 M-GuHCl, pH 7.0, for molecular weight (M.W.) determinations. Both the low speed and high speed meniscus depletion sedimentation equilibrium methods of molecular weight determination, as described by Chervenka (1969), were used. In order to shorten the transient period before reaching equilibrium in low-speed runs, an initial overspeeding of the rotor at approximately twice the estimated equilibrium speed was employed. The appropriate rotor speed in relation to the expected molecular weight of the protein under examination was obtained from the selection chart of Chervenka

(1969). The distribution of material in the centrifuge cell was determined with a photo-electric scanning system and was judged to be in equilibrium when successive scans at 3 h intervals revealed no further change in the absorbance profile.

A six-channel Yphantis centrepiece, sapphire windows and either the An-D or An-J rotors were routinely used for molecular weight determinations. All sedimentation equilibrium runs were scanned two or three times at two different rotor speeds and at three protein concentrations, to test for homogeneity of compounds.

Analysis of the photo-electric scanner recordings were done as described by Chervenka (1969). Molecular weights were calculated as follows:

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \times \frac{d \ln c}{d(x^2)}$$

where R = gas constant;

T = absolute temperature;

\bar{v} = partial specific volume;

ρ = density of solution;

x = distance from axis of rotation;

ω = angular velocity;

c = concentration (absorbance units).

Apparent molecular weights were calculated by computer from the regression analysis of plots of $\ln c$ versus x^2 . The computer programme employed and examples of a data input sheet and the printout of a completed calculation is shown in Appendix 2. The density, at different temperatures, of a 6 M-GuHCl solution

was calculated by the equation of Kawahara and Tanford (1966) and was verified pycnometrically:

$$\frac{d}{d_0} = 1 + 0.2710 W + 0.0330 W^2$$

where d = density of the solution;

d_0 = density of water;

W = weight fraction of GuHCl in the solution.

Partial specific volumes used (\bar{v}) were those published by Dorrington and Mihaesco (1970).

5.3.4 Circular dichroism measurements

Circular dichroism (CD) spectra were recorded on a Jasco model J-20 automatic recording spectropolarimeter at 18°C. The signal to the recorder pen was simultaneously fed through a Beckman Auto Pro 311 analogue-to-digital converter to an ITT tele-typewriter-punch, which collected the data in ASCII-coded form on paper tape for subsequent off-line computerized analysis. Cylindrical fused quartz cells with pathlengths of 0.1 and 1.0 cm were used. Proteins were dissolved in and dialysed overnight against 0.075 M-phosphate buffer, pH 7.0. Such protein solutions were diluted, if necessary, to an absorbancy of between 1 and 2 and their concentrations (mg/ml) calculated from the extinction coefficients ($E_{280\text{nm}}^{1\%}$) of 13.4 and 13.1 previously determined (chapter 2) for normal IgM and IgM(Sad).

The mean residue ellipticities $[\theta]$ were calculated by an IBM 1130 computer from the data on the paper tapes according to the equation:

$$[\theta] = \frac{\theta_{\text{obs}} \times 100 \times M}{l \times c} \text{ deg.cm}^2.\text{dmole}^{-1}$$

where θ_{obs} = observed ellipticity in degrees;
 M = amino acid mean residue weight, taken as
 108 (Crumpton and Wilkinson, 1963);
 l = path length (cm);
 c = concentration of protein (mg/ml).

The calculated values were plotted on a Ca'comp 563 incremental graph plotter and the points connected by the best-fitting smooth line (calculated from the experimental values by a five-point quadratic smoothing procedure) to yield the CD spectra. The computer programmes employed for these manipulations are listed in Appendix 3.

5.4 RESULTS AND DISCUSSION

5.4.1 Amino acid analysis

5.4.1.1 Amino acid analysis of the fast anodal component (FAC, fraction 14c) isolated from a tryptic digest of normal IgM. The amino acid composition of fraction 14c is given in Table 3.

The high content (26% of amino acid residues, Table 3) of aspartic and glutamic acid in FAC (fraction 14c) is adequate explanation for the fast anodal mobility of this minor component (Plates 13a and 15c). Only 36.6% of the weight of the hydrolysed sample of FAC was recoverable as amino acids, and unidentified peaks appeared on the chromatograms in positions corresponding to those of amino sugars. It is surmised that this fragment probably stems from the Fc_{μ} part of normal IgM. $Fc_{5\mu}$ is known to contain more carbohydrate than the Fab_{μ}

Table 3. Amino acid composition of fraction 14c. (Means of duplicate 24-h hydrolysates).

Amino acid	moles residue/10,000 g protein
Lys	1.87
His	0.86
Arg	1.29
Asp	9.43
Thr	5.40
Ser	7.41
Glu	10.65
Pro	6.19
Gly	5.47
Ala	4.38
$\frac{1}{2}$ - Cys	5.61
Val	2.88
Met	1.44
Ile	1.73
Leu	4.53
Tyr	7.05
Phe	1.73
	<hr/> 77.92 <hr/>

fragment of the IgM molecule (Davie and Osterland, 1971; Putnam et al., 1972). Perusal of the amino acid sequence of the hinge region of IgM(0u) (Fig. 22) reveals that this part of the μ -chain contains 7 dicarboxylic amino acid residues ($\frac{7}{21} = 33.3\%$) as well as one half-cystine residue and a carbohydrate moiety. The anodal mobility of FAC and the characteristic presence of dicarboxylic amino acid and half-cystine residues as well as the occurrence of carbohydrate moieties in both FAC and the hinge region of IgM(0u) may perhaps be taken to indicate that fraction 14c represents this region of the μ -chain of normal IgM.

5.4.1.2 Fractions 17a and 17c (normal Fabu). The object of this analysis was to determine the relative acidic and

basic amino acid content of the two normal Fabu species which were separated from each other by means of DEAE-cellulose chromatography (Fig. 17).

Table 4. Relative amino acid composition of two differently charged species of normal Fabu (fractions 17a and 17c).

Amino acid	fraction 17a (R_{Leu})	fraction 17c (R_{Leu})
Lys	0.69	0.62
His	0.19	0.24
NH ₃	1.92	1.74
Arg	0.46	0.41
Asp	1.04	1.32
Thr	1.00	1.29
Ser	1.58	2.02
Glu	1.04	1.33
Pro	0.65	0.91
Gly	0.96	1.09
Ala	0.81	0.94
$\frac{1}{2}$ - Cys	trace	0.13
Val	0.96	1.09
Met	trace	0.13
Ile	0.31	0.39
Leu	1.00	1.00
Tyr	0.5	0.55
Phe	0.38	0.45

It is clear that fraction 17c had a higher content of negatively charged residues (Asp and Glu) and a lower content of positively charged residues (Arg and Lys) than fraction 17a, in accordance with their relative order of elution upon separation on a DEAE-anion exchanger.

In order to show the difference in charge between fractions 17a and 17c better, the sums of the relative values for the acidic (Glu and Asp) and basic amino acids (Lys and Arg) in Table 4 are compared in Table 5.

Table 5. Comparison of the content of acidic and basic amino acids in fractions 17a and 17c.

Amino acids	Relative charge ^a	
	fraction 17a	fraction 17c
Basic	+ 1.15	+ 1.03
Acidic	$-(2.08-0.18) = -1.90^b$	-2.65
Nett relative charge	-0.75	-1.62

a: At pH 8.0 used for elution from a DEAE anion exchanger.

b: Corrected for the higher relative NH_3 content of 17a, assuming that this would be due to a higher asparagine and glutamine content of this fraction.

The difference in the nett relative charges of fractions 17c and 17a, with the former species being more negatively charged by $(1.62-0.75) = 0.87$ relative units, explains the ease of separation of these two normal Fab_μ species by means of anion exchange chromatography.

5.4.1.3 Fractions 19c and 24b ($\text{Fc}_\mu(\text{Sad})$). The production of a small peptide (fraction 19c) by high temperature tryptic digestion of $\text{IgM}(\text{Sad})$ and its immunological identification as a subfragment of $\text{Fc}_{5\mu}(\text{Sad})$ (Plate 28), necessitated a comparison of its amino acid composition with that of intact $\text{Fc}_\mu(\text{Sad})$. If fraction 19c were formed with equal facility from both the amino- and carboxy-terminal halves of $\text{Fc}_\mu(\text{Sad})$, it could be predicted that their respective amino acid compositions should reveal little or no difference. The results in Table 6, however, show big differences for instance in respect of glycine, serine and half-cystine, between fraction 19c and $\text{Fc}_\mu(\text{Sad})$.

Table 6. Relative amino acid composition of fraction 19c and Fc μ (Sad) (fraction 24b).

Amino acid	fraction 19c (R_{Leu})	Fc μ (Sad) (R_{Leu})
Lys	0.23	0.39
His	0.25	0.29
Arg	0.11	0.58
Asp	1.68	1.12
Thr	1.69	1.62
Ser	2.06	1.10
Glu	1.43	1.37
Pro	0.93	0.99
Gly	1.33	2.69
Ala	1.18	0.94
$\frac{1}{2}$ - Cys	0.78	0.15
Val	1.65	1.10
Met	0.21	0.18
Ile	0.35	0.48
Leu	1.00	1.00
Tyr	0.34	0.34
Phe	0.88	0.77

If the number of half-cystine residues in the amino acid sequence of Fc(0u) and in its amino-terminal half (Fig. 22) is expressed relative to their respective leucine contents, it is found that they occur at almost twice the frequency in the latter subfragment. The preponderance of this particular amino acid in the amino-terminal half of Fc(0u) is due to the presence of two or possibly three disulphide bridges in this region of the IgM(0u) molecule (Putnam *et al.*, 1972). It would therefore appear possible that fraction 19c might represent part of the amino-terminal half of Fc μ (Sad).

5.4.2 Ultracentrifugation analysis

Sedimentation coefficients determined for normal IgM, IgM(Sad) and the respective reductive subunits and tryptic fragments of these immunoglobulins are given in Table 7. The molecular weights

of these proteins and their respective μ -chains are shown in Table 8.

Table 7. Sedimentation coefficients ($S_{20,w}^0$) with standard deviations determined for normal IgM and IgM(Sad) and their respective derivatives.

Species	Sedimentation coefficient		
	Normal IgM	IgM(Sad)	Literature values
IgM	$16.86S \pm 0.11$	$17.74S \pm 0.12$	17.90S (1) 18.94S (2)
IgM _s	—	$7.10S \pm 0.20^a$ $6.90S \pm 0.03^b$	7.07S (1) 6.82S (2)
Fab μ	$3.58S \pm 0.02^c$ $3.40S \pm 0.04^d$	$3.63S \pm 0.08$	3.7S (3) 3.59S (4)
Fc ₅ μ	$10.01S^e$	$10.81S \pm 0.15$	10.8S (3)
Fc μ	—	$3.06S \pm 0.02$	3.4S (3)

a: IgM_s obtained after reduction with 0.01 M DDT and alkylation

b: IgM_s obtained after reduction with 0.015 M MEA and alkylation

c: Fraction 17a (Chapter 3)

d: Fraction 17c (Chapter 3)

e: Determined on a solution with $A_{280 \text{ nm}}^{1 \text{ cm}} = 4.80$

- References:
- 1) Miller and Metzger (1965a).
 - 2) Suzuki and Deutsch (1967).
 - 3) Plaut and Tomasi (1970).
 - 4) Beale and Butters (1969).

Table 8. Weight-average molecular weights^a (\bar{M}_w^O) with standard deviations in 6 M-GuHCl of normal IgM and IgM(Sad) and their respective derivatives.

Species	Average molecular weights		
	Normal IgM	IgM(Sad)	Literature values
	$\bar{M}_w^O \times 10^{-3}$	$\bar{M}_w^O \times 10^{-3}$	$\bar{M}_w^O \times 10^{-3}$
IgM	842.0 ± 30.6^c	987.6 ± 27.7^c	891.0 ± 20.6 1
	640.3 ± 40.3^c	$993.4^{c,d}$ —	845.0 2
			890.0 3
IgM _s	—	$162.6 \pm 1.7^{d,f}$ $151.9^{d,e}$	185.0 4
Fab μ	47.3 ± 1.2	47.4 ± 1.4	41.0 2
			47.0 3
			46.6 5
Fc ₅ μ	326.6 ± 24.4^c	283.8 ± 14.1^c	314.3 ± 6.2^g 1
		311.5 ± 16.8^c	342.0 2
		329.5 ± 17.3^c	320.0^g 6
monomeric Fc μ	35.6 ± 0.8	31.7 ± 1.7	31.5 ± 1.1^g 1
			33.7^b 2
μ -chain	63.6 ± 1.3	61.8 ± 1.6	65.2 ± 1.8 1

a: \bar{M}_w^O were determined from at least 12 individual scans

b: This is half the molecular weight of 67,300 daltons determined under non-dissociating conditions (ref. 2)

c: Molecular weight values showed concentration and/or rotor speed dependence

d: Determined in 0.05 M-Tris-0.5 M-NaCl buffer, pH 8.0

e: Reduced with 0.01 M DTT and alkylated

f: Reduced with 0.015 M MEA and alkylated

g: From papain digests.

- References:
- 1) Dorrington and Mihaesco (1970).
 - 2) Plaut and Tomasi (1970).
 - 3) Miller and Metzger (1966).
 - 4) Miller and Metzger (1965a)
 - 5) Beale and Buttress (1969).
 - 6) Onoue et al. (1968).

5.4.2.1 Theoretical considerations. The problem of aggregation of μ - and L-chains and immunoglobulins in neutral aqueous solutions, has the consequence that reliable molecular weights can only be obtained in dissociating solvents. Sedimentation equilibrium methods of M.W. determination provides estimates of $M(1 - \rho\bar{v})$, where ρ is the solution density and \bar{v} the partial specific volume of the protein under investigation. The accuracy of \bar{v} plays an inordinately big role in the determination of M.W. in 6 M-GuHCl solution, and a 1% error ($= \pm 0.007$ ml/g) in this constant will introduce an uncertainty in the M.W. of 5 - 6%. Although a reasonable estimate of \bar{v} for protein in dilute salt solutions may be obtained from the \bar{v} of the constituent amino acids (Cohn and Edsall, 1943), such an average \bar{v} does not take into account the higher order of structure inherent in native protein molecules or the effect that this may have on \bar{v} . For glycoproteins a further correction must be applied for the lower relative contribution in \bar{v} made by the carbohydrate moiety and an additional correction is also necessary to compensate for the preferential binding of GuHCl to protein (Hade and Tanford, 1967). In the present investigation, the \bar{v} -values used for normal IgM, IgM(Sad) and the various tryptic fragments and reduced and alkylated polypeptides, were taken from Dorrington and Mihaesco (1970). These values were obtained from amino acid

and carbohydrate analyses and corrected for preferential GuHCl binding by subtraction of 0.01 ml/g. It is conceded that this approach could have introduced some uncertainty as to the absolute values of the molecular weights in Table 8.

5.4.2.2 Molecular weights of normal IgM and IgM(Sad). The molecular heterogeneity and semi-fragmented nature of some normal IgM preparations (Plate 36) has already been mentioned and it was speculated that plasmin may have caused slow fragmentation during the isolation process of normal IgM from Cohn fraction III (chapter 4). The low value of $S_{20,w}^O = 16.9S$ (Table 7) determined for one such preparation was therefore not surprising. For these reasons, normal IgM and IgM(Sad) were rechromatographed on Sepharose 6B under dissociating conditions to ensure that preparations of higher homogeneity would be used for molecular weight determinations. SDS-PAGE analysis of the resulting preparations is shown in Plate 42.

The molecular weight (M.W.) of 845.0×10^3 daltons determined for normal IgM (Table 8) is in agreement with the accepted value of about 890.0×10^3 daltons for Waldenström IgM (Miller and Metzger, 1965a ; Dorrington and Mihaesco, 1970). It is relevant that molecular weights ranging over $620.0 \times 10^3 - 120.0 \times 10^4$ daltons have also been reported in the literature (Fillitti-Wurmser and Hartmann, 1968). The heterogeneity of all normal IgM preparations used for M.W.-determinations was evident from the concentration and/or rotor speed dependence shown by these preparations.

Although the $S_{20,w}^O = 17.7S$ determined for IgM(Sad) (Table 7)

was comparable to that found by Miller and Metzger (1965a), the molecular weight of this preparation, approximately 1×10^6 daltons (Table 8), is about 15% higher than that of the Waldenström IgM investigated by them and is more in line with the results of Suzuki and Deutsch (1967). The slight molecular heterogeneity indicated by a rotor speed dependence of the M.W.-values of IgM(Sad) could be ignored, in the light of a standard deviation of only 2.8% (see for example Dorrington and Mihaesco, 1970).

5.4.2.3 Molecular weights of normal Fab μ and Fab μ (Sad).

The molecular weights found for normal Fab μ (47.3×10^3 daltons, Table 8) and Fab μ (Sad) (47.4×10^3 daltons, Table 8) are in good agreement with the literature values reported for Fab μ isolated from the 37°C tryptic digests of Waldenström IgM's (Miller and Metzger, 1966; Beale and Buttress, 1969). The rather low value of 41,000 daltons reported by Plaut and Tomasi (1970) for Fab μ obtained from a 56°C tryptic digest could be rationalised if it is taken into account that in the case of IgM(Ou) (Putnam et al., 1972), the inter- μ -L-chain disulphide bridge joining these two chains is found at a position about halfway along the Fd μ -fragment (position 140 - Fig. 22). If trypsin cleavage occurred not only at the usual arginine (217) position, but also at Lys-168 and Lys-201, a glycopeptide with a M.W. of several thousand daltons would be "scooped out" of Fab μ ; this would not affect the latter's disulphide-linked two-chain structure, but would yield an Fab μ -species with a M.W. appreciably lower than the usual value of

about 47,000 daltons. Calculation of the M.W. of the F_{μ} -piece shown in Fig. 22 (23,900 daltons) and addition of 23,500 daltons for L-chain gives a molecular weight of 47,400 daltons for Fab_{μ} of IgM(0u). This value can be regarded as reasonable for a lower M.W. of Fab_{μ} , formed by trypsin cleavage at a single point only, since the residue weight of each individual amino acid is taken into account in such a calculation and since Putnam et al., (1972) stated that the F_{μ} -chain might be slightly longer.

5.4.2.4 Molecular weights of normal $F_{C5\mu}$ and $F_{C5\mu}(Sad)$. The variable M.W. of $F_{C5\mu}(Sad)$, where differences of up to 14% were observed amongst different preparations, the large standard deviation for the M.W. of normal $F_{C5\mu}$ and their rotor speed and concentration dependence (Table 8) reflect on the heterogeneity of these fragments. This observation was of course not unexpected in view of the heterogeneity observed earlier in SDS-PAGE analysis of normal $F_{C5\mu}$ (Plate 17) and $F_{C5\mu}(Sad)$ (Plate 25). The mean molecular weights for normal $F_{C5\mu}$ and $F_{C5\mu}(Sad)$ are however comparable to those reported in the literature (Dorrington and Mihaesco, 1970; Plaut and Tomasi, 1970; Onoue et al., 1968). It is significant that the molecular weights found for the respective reduced and alkylated monomers of normal $F_{C5\mu}$ and $F_{C5\mu}(Sad)$ in GuHCl were approximately one tenth that of the polymeric F_{C5} precursors. This provides evidence that normal IgM can also be represented by the pentameric ($5 \times IgM_5$) model proposed by Miller and Metzger (1965b) and is in agreement with the results of Plaut and Tomasi, (1970) and

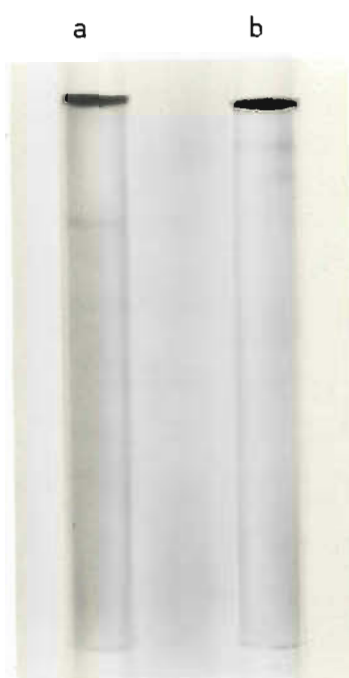


Plate 42 SDS-PAGE (5.6% gel) analysis of (a) normal IgM and (b) IgM(Sad) after chromatography on a column of Sepharose 6B equilibrated with 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0.

Dorrington and Mihaesco, (1970). Similarly, the monoclonal immunoglobulin of the present investigation (IgM(Sad)) also conforms to this model.

5.4.2.5 Molecular weights of the μ -chains of normal IgM and IgM(Sad). The molecular weights of the μ -chains (Table 8) for both normal IgM (63,600 daltons) and IgM(Sad) (61,800 daltons) are slightly lower than those reported in the literature (Metzger, 1970), but are considered to be accurate values in view of their high degree of purity on SDS-PAGE (Plate 38) and their low standard deviations (<3%).

5.4.2.6. Molecular weight of IgM_S(Sad). The M.W. determined for the reduced and alkylated subunit (IgM_S) of IgM(Sad) is much lower than the usual literature values of about 185,000 daltons (Metzger, 1970). The figure of 151,900 daltons shown in Table 8 for IgM_S(Sad), is the mean of 54 determinations on 3 different preparations of IgM_S(Sad) obtained by reduction with 0.01 M-DTT, alkylation and column chromatographic purification. This M.W. agrees with that calculated for a structure consisting of two μ -chains, but only one L-chain, in analogy with the results of Suzuki and Deutsch, (1967). These authors proposed that reduction of IgM without any subsequent alkylation, produces an IgM_S consisting of 2 μ -chains + 3 L-chains and with a sedimentation coefficient of 8-S. If reduction is followed by alkylation (the normal procedure to obtain IgM_S) the 8-S species loses 2 of its L-chains yielding a 7-S molecule consisting of 2 μ -chains, but only 1 L-chain. In an extensive study they obtained strong evidence for such a

model from amino acid analysis, column chromatographic yield studies of μ - and L-chain, ultracentrifugation analysis and also proteolytic fragmentation studies (Suzuki, 1969). Their work received support from the results of Chen et al. (1969), but there appears to be a general problem in the determination of the true molecular weight of reduced and alkylated IgM_s (Beale and Buttress, 1969; Chen et al., 1969). It has recently been suggested that this is probably caused by the phenomenon of "longitudinal" dissociation of IgM_s into HL-halves at low protein concentrations (Egerov et al., 1971).

In order to avoid complications due to dissociation, IgM_s (Sad) was also prepared according to the method of Morris and Inman (1968). These authors claimed that reduction with 2-mercaptoethylamine (0.015 M), rather than DTT, gives IgM_s with intact inter 7-S interchain disulphide bonds (i.e. inter- μ -L- and inter- μ - μ -chain). The molecular weight (162,600 daltons) obtained on such a preparation (Table 8) did show an increase above the value (151,900 daltons) found with DTT as reducing agent, but still fell far short of that expected (185,000-200,000 daltons) for a conventional type of IgM_s derived from the parent IgM(Sad) with a M.W. close to 1×10^6 daltons.

Because of the unsatisfactory and perplexing results obtained with IgM_s (Sad) and because of the known ultracentrifugal polydispersity of normal IgM_s (Plate 39b), no effort was made at M.W. determination on the latter molecule.

5.4.2.7 Reconstitution studies. It now remained to be

established whether or not the molecular weights of the two parent IgM molecules under investigation could be reconstituted from those of their components, using an acceptable stoichiometry. Such a procedure carried out on a Waldenström IgM is to be found in the publication of Dorrington and Mihaesco (1970) (see Fig. 7). The results of a similar exercise on the normal IgM species isolated in the present investigation are presented in Table 9.

Table 9. Reconstitution of the molecular weight of normal IgM from molecular weights determined for its constituent μ - and L-chains or Fab μ and monomeric Fc μ fragments.^a

Reduced and alkylated polypeptide chains	Tryptic fragments
10 x μ -chain = 636,000	10 x Fab μ = 473,000
10 x L-chain = 230,000 ^c	10 x monomeric Fc μ = 356,000
866,000	10 x hinge region ^b = 44,000
	873,000

a: based on a pentameric structure consisting of 10 μ - + 10 L-chains.

b: calculated from the hinge region amino acid sequence of IgM(0u) (Paul *et al.*, 1971).

c: mean M.W. of κ - and λ -chains calculated from amino acid sequence analysis (Putnam *et al.*, 1967).

The two calculated values, derived in different fashion, for the M.W. of normal IgM are in good agreement with each other and also with that experimentally observed (842,000) for the intact molecule (Table 9) and literature values (Metzger, 1970) (The addition of 44,000 daltons to account for the hinge region of IgM which is extensively digested by trypsin, is considered to be a justifiable manipulation). These results therefore

established that the molecular structure of normal IgM also conforms to the well-known pentameric, 10 μ - + 10 L-chain model proposed for most Waldenström IgM's.

A similar calculation (Table 10) for IgM(Sad) does not, however, present a comparably clear-cut picture.

Table 10. Reconstitution of the molecular weight of IgM(Sad) from molecular weights determined for its constituent μ - and L-chains or Fab μ and monomeric Fc μ fragments.^a

Reduced and alkylated polypeptide chains	Tryptic fragments
10 x μ -chain = 618,000	10 x Fab μ fragment = 474,000
10 x L-chain = 226,000 ^c	10 x monomeric Fc μ = 317,000
<u>844,000</u>	10 x hinge region ^b = 44,000
	<u>835,000</u>

a: based on a pentameric structure consisting of 10 μ - + 10 L-chains.

b: calculated from the hinge region amino acid sequence of IgM(Ou) (Paul *et al.*, 1971).

c: calculated from the amino acid sequence analysis of λ -chain (Putnam *et al.*, 1967).

Although the internal consistency of the two calculated molecular weights is good and also in agreement with literature values (Metzger, 1970) they differ significantly from the M.W. actually determined. Addition of 5 more L-chains ($5 \times 22,600 = 113,000$ daltons) to those shown in Table 10, i.e. assuming a 10 μ - + 15 λ -chain model, yields a M.W. of approximately 960,000 daltons which is in much better agreement with the observed value of approximately 990,000 daltons (Table 8). Moreover, densitometric scans of the acrylamide gel cylinder shown in Plate 37 (reduced and alkylated IgM(Sad)) gave a weight distribution of 68% and 32% for μ - and L-chains

respectively, which coincides exactly with the values reported by Suzuki and Deutsch (1967) and on which they based their $10 \mu + 15$ L-chain model. It would therefore appear that IgM(Sad) could be another example of a $10 \mu + 15$ L-chain variant. This raises the possibility that there actually exist two species of IgM which are differentiated only by their L-chain content: one type having 10 L-chains, as for normal IgM and probably the majority of Waldenström immunoglobulins, and the other having 15 L-chains and perhaps found only rarely amongst monoclonal IgM's.

Overproduction of L-chain and the appearance of this protein in urine was first described by Bence-Jones (1847) and is a well-known phenomenon in some patients suffering from the production of abnormal immunoglobulins. Bence-Jones protein was in fact detected in the urine of the patient from whose plasma IgM(Sad) was isolated. Immunoglobulin M biosynthesis in a mouse plasma-cell tumour has been extensively studied by Parkhouse (1971). This tumour produces L-chain in excess of μ -chain and it was established that a large pool of L-chain participates in the intracellular biosynthesis of μ L and μ_2 L₂ (IgM_s) molecules. Furthermore, it has been determined (Askonas and Parkhouse, 1971) that for this tumour, the order of IgM-assembly is $\mu + L \rightarrow \mu L \rightarrow \mu_2 L_2 \rightarrow (\mu_2 L_2)_5$. Because the first step in assembly is $\mu + L \rightarrow \mu L$, one could speculate that in some cases of abnormal immunoglobulin synthesis where L-chain is overproduced, more than one L-chain could become covalently linked to a μ -chain. The Fd-part of the μ -chain

appears a likely area onto which to link such an additional L-chain via a cystine residue normally involved in an intra- μ -chain disulphide bond. The product of such an assembly would be μL_2 , which would conceivably couple more readily with the sterically less hindered usual intermediate, μL , than with another μL_2 , to form a $\mu_2 L_3$ entity. A pentameric molecule built up from these $\mu_2 L_3$ subunits would then conform to the 10 μ - + 15 L-chain model of Suzuki and Deutsch (1967).

5.4.3. Circular dichroism spectra

5.4.3.1 Normal Fab μ and Fab μ (Sad). Trypsin digestion of normal IgM yielded two species of Fab μ which could be separated on a DEAE-cellulose column (chapter 3.4.4) whereas IgM(Sad) yielded only a single Fab μ entity (chapter 3.4.7).

A comparison of the CD spectra of these Fab μ -fragments (Figs. 29-34) revealed similarities but also some differences in their optical properties. The large negative ellipticity band at 217-218 nm in the CD spectra of both normal Fab μ and Fab μ (Sad) (Figs. 29, 31 and 33) is characteristic for the immunoglobulins of most animal species (Litman et al., 1971a), their proteolytic fragments (Cathou et al., 1968; Ashman et al., 1971; Ghose, 1971), γ -chains (Björk and Tanford, 1971a) L-chain (Björk and Tanford, 1971b) and the carboxy- and amino-terminal halves of λ -chain (Björk et al., 1971). Notable exceptions are the immunoglobulins of the sea lamprey (Litman et al., 1971a), the carboxy-terminal half of Fc γ (Litman et al., 1971b) and fraction 19c described below. The mean residue

ellipticity of this band for both normal and monoclonal Fab μ -fragments investigated (2,000-3,000 deg. cm². dmole⁻¹) is of the same order of magnitude as reported in the literature (Ashman et al., 1971; Litman et al., 1971a).

It has been suggested (Sarkar and Doty, 1966; Cathou et al., 1968) that the 217-218 nm CD band is due to the presence of β -conformation in these proteins. A negative ellipticity band at this wavelength was predicted from calculations of the ultraviolet optical properties of polypeptides in β -conformation and it is thought to result from a $n \rightarrow \pi^*$ transition of the lone pair of electrons of oxygen (Pysh, 1966). Support for this has also come from the demonstration of such a band in model peptides such as poly-L-lysine (Townend et al., 1966) and other proteins with known β -conformation (Iizuka and Yang, 1968; Lederer, 1968). On the other hand, Cathou et al., (1968) observed that for rabbit Fab γ , this band persisted in the presence of 6 M-GuHCl. This is clearly in contrast with the findings of Tanford et al. (1967a, 1967b) who have concluded that proteins exist essentially as random chains in 6 M-GuHCl even though a completely random orientation may be prevented by the presence of intrachain disulphide bonds.

The positive ellipticity band at 235-236 nm (Figs. 29, 31 and 33) is very interesting in that, although quite prominent in fraction 17a (normal Fab μ , Fig. 29) it is almost absent in fraction 17c (normal Fab μ , Fig. 31) and fraction 19b (Fab μ (Sad), Fig. 33). This band is thought to be due to rotationally restricted tyrosine side chains (Cathou et al.,

1968). Similar bands have been observed for the Fab μ I and Fab μ II fragments isolated from rabbit IgG (Cathou et al., 1968) and also for human IgG and 7-S IgM (Litman et al., 1971b), rabbit IgG (Björk and Tanford, 1971c), rabbit L-chain dimer (Björk and Tanford, 1971b) and also for the V- but not the C-region of human λ -chain (Björk et al., 1971). The finding of the last-mentioned authors can possibly be interpreted to reflect on the proposed role which tyrosine plays in the antigen-binding site situated in the V $_L$ and V $_H$ regions of immunoglobulins (Singer and Thorpe, 1968; Franěk., 1971). The fact that the Fab μ -fragments of IgM(Wag) (Ashman et al., 1971) and IgM(0u) (Putnam et al., 1972) contain more tyrosine residues than their respective Fc μ -fragments may also be considered to support this hypothesis. Cathou et al. (1968) have found however, that rabbit anti-DNP Faby I and II fragments gave the same CD spectra whether the hapten was present or not.

Björk and Tanford (1971b) made the interesting observation that isolated L-chain, existing in dimeric form, showed the presence of this positive ellipticity band (237 nm) whilst for its monomeric counterpart, only a shoulder could be detected at this wavelength. They speculated that dimerisation of L-chain may cause the more prominent appearance of this band, but also reasoned that it is possible that dimerisation is perhaps an intrinsic property of different species of L-chain and that this property is merely reflected in its CD spectrum. That L-chain dimerisation has nothing to do with L-chain class (i.e. κ or λ) may be deduced from the fact that both monomers

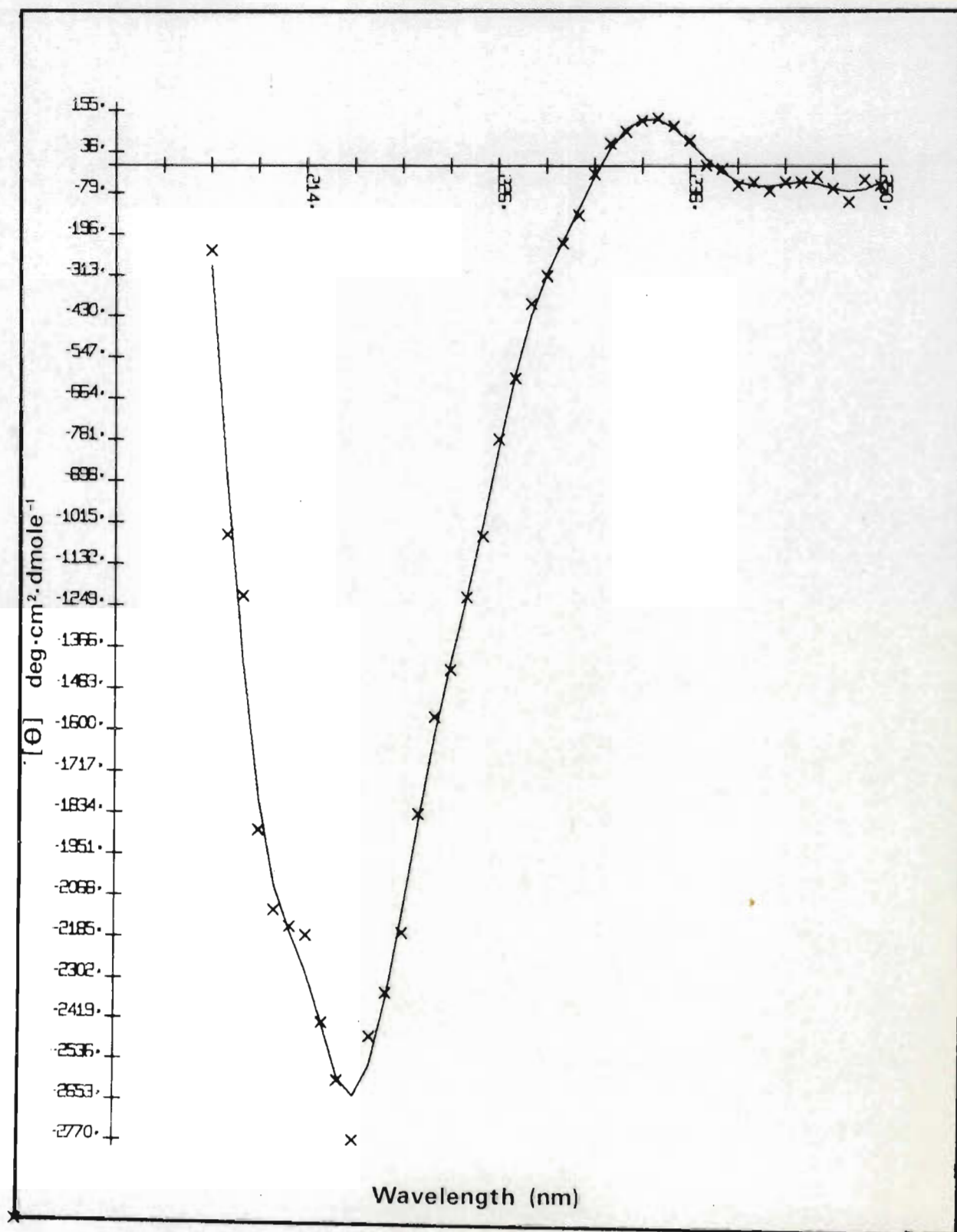


Fig. 29: CD spectrum (205-250 nm) of normal Fabu (fraction 17a) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 1.103 mg/ml)

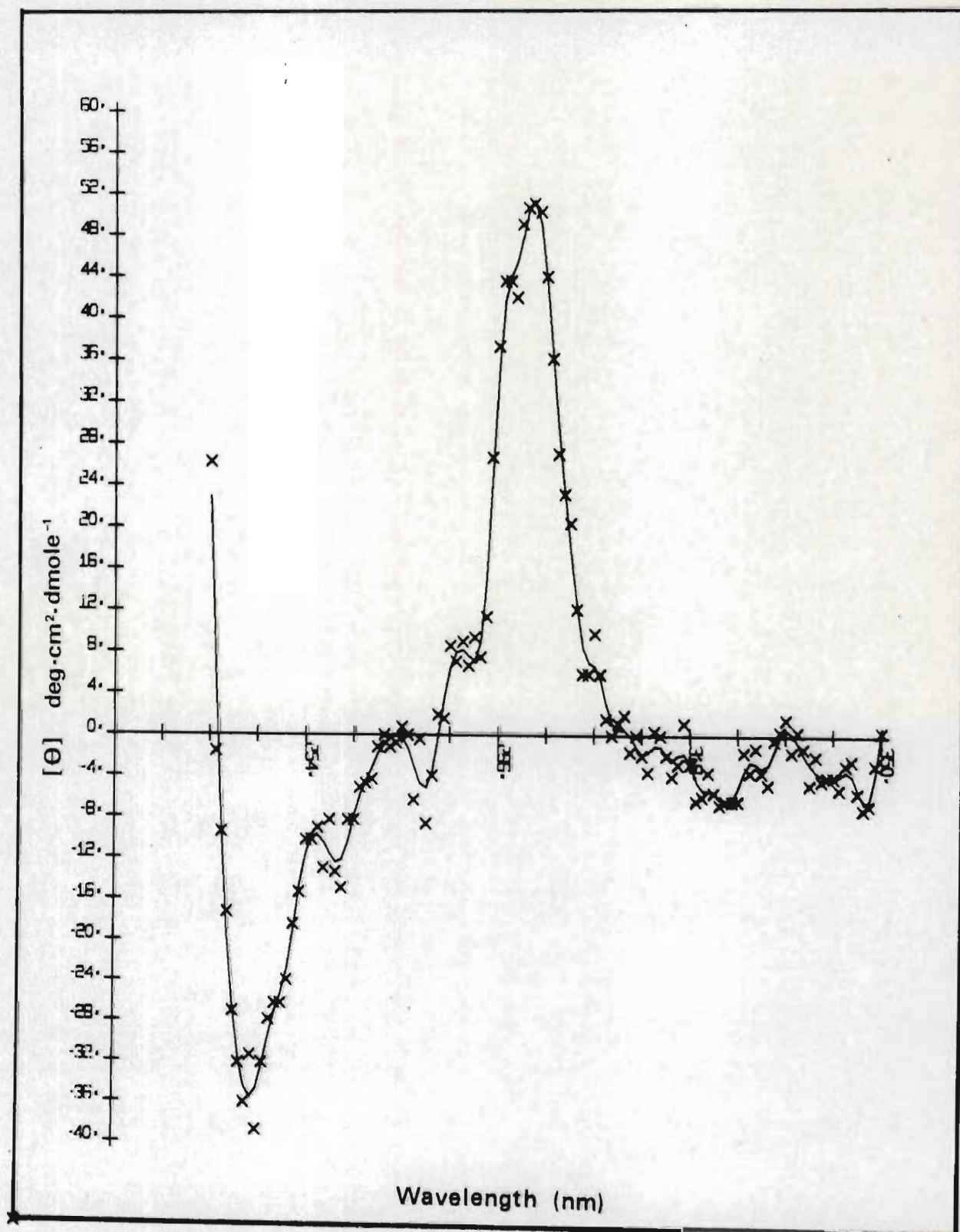


Fig. 30: CD spectrum (230-350 nm) of normal Fabu (fraction 17a) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 1.103 mg/ml)

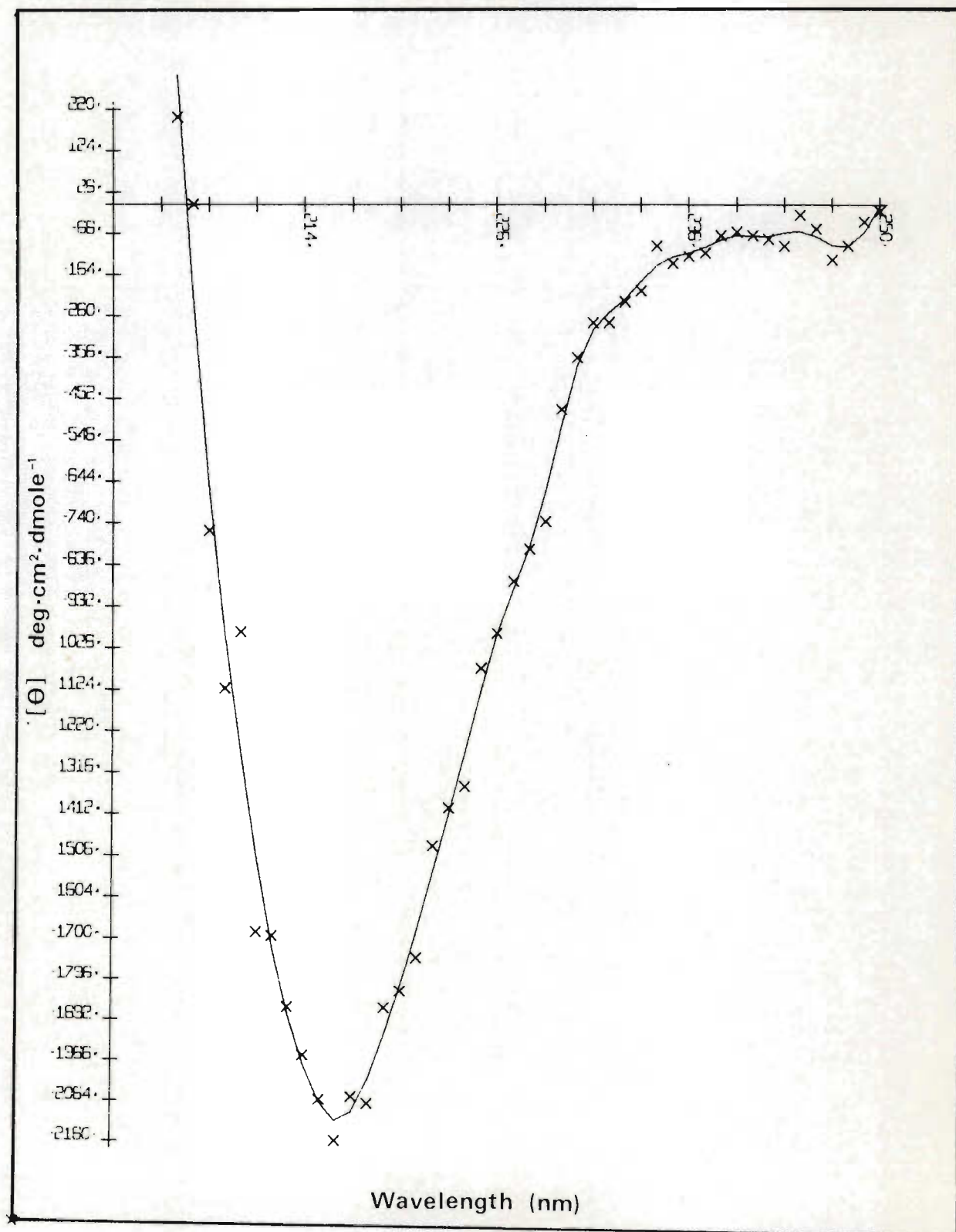


Fig. 31: CD spectrum (205-250 nm) of normal Fab μ (fraction 17c) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 1.073 mg/ml)

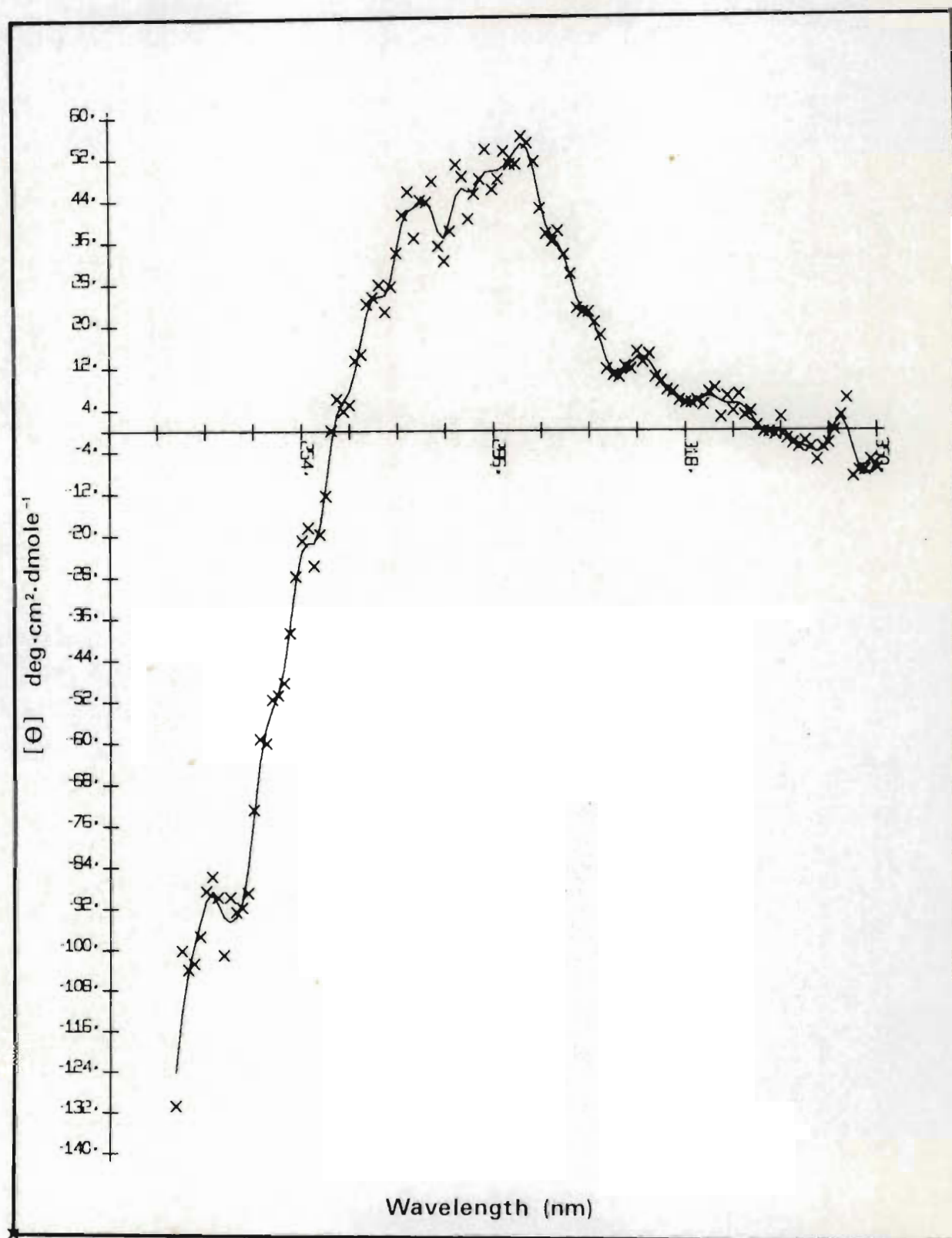


Fig. 32: CD spectrum (230-350 nm) of normal Fabu (fraction 17c) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 1.073 mg/ml)

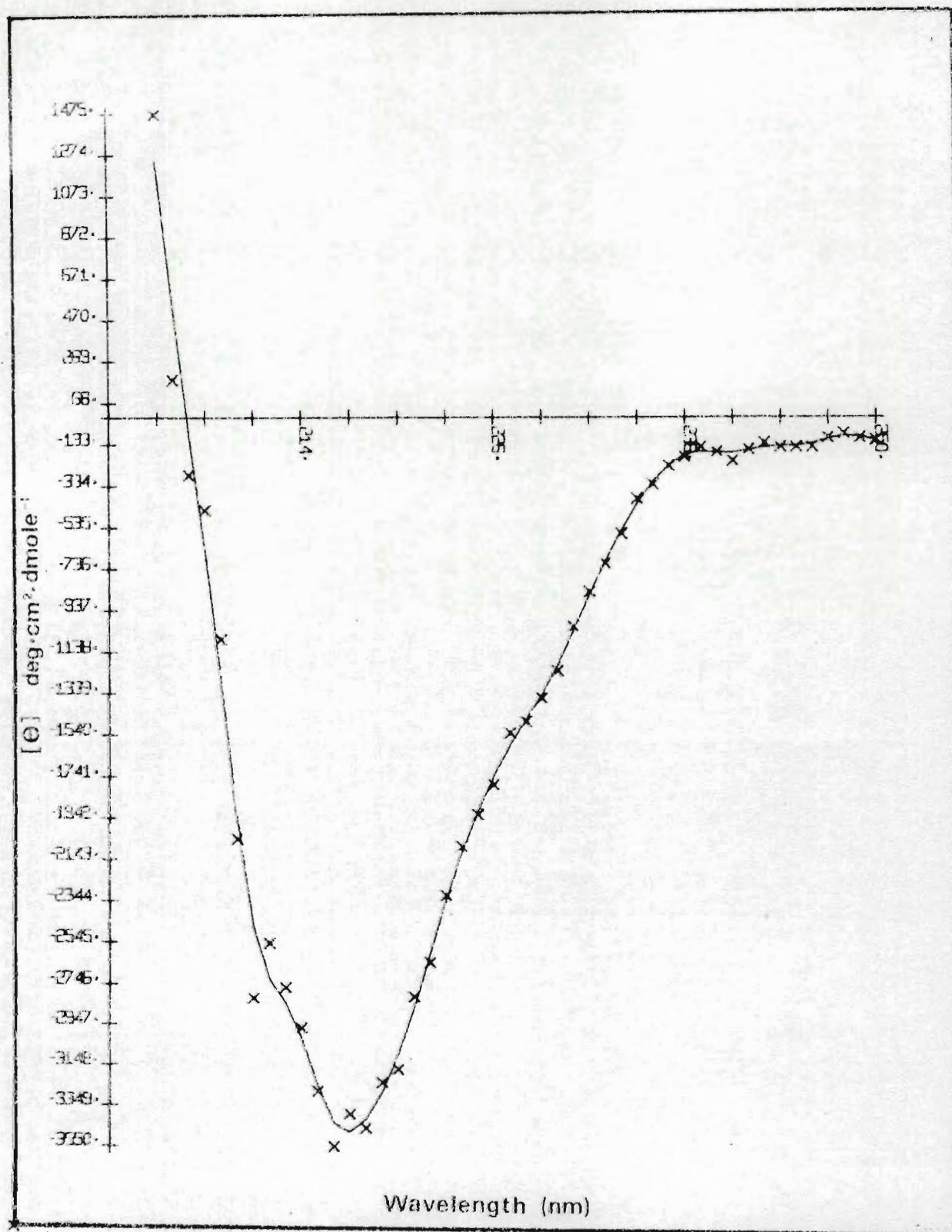


Fig. 33: CD spectrum (205-250 nm) of Fab μ (Sad) (fraction 21a) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.770 mg/ml)

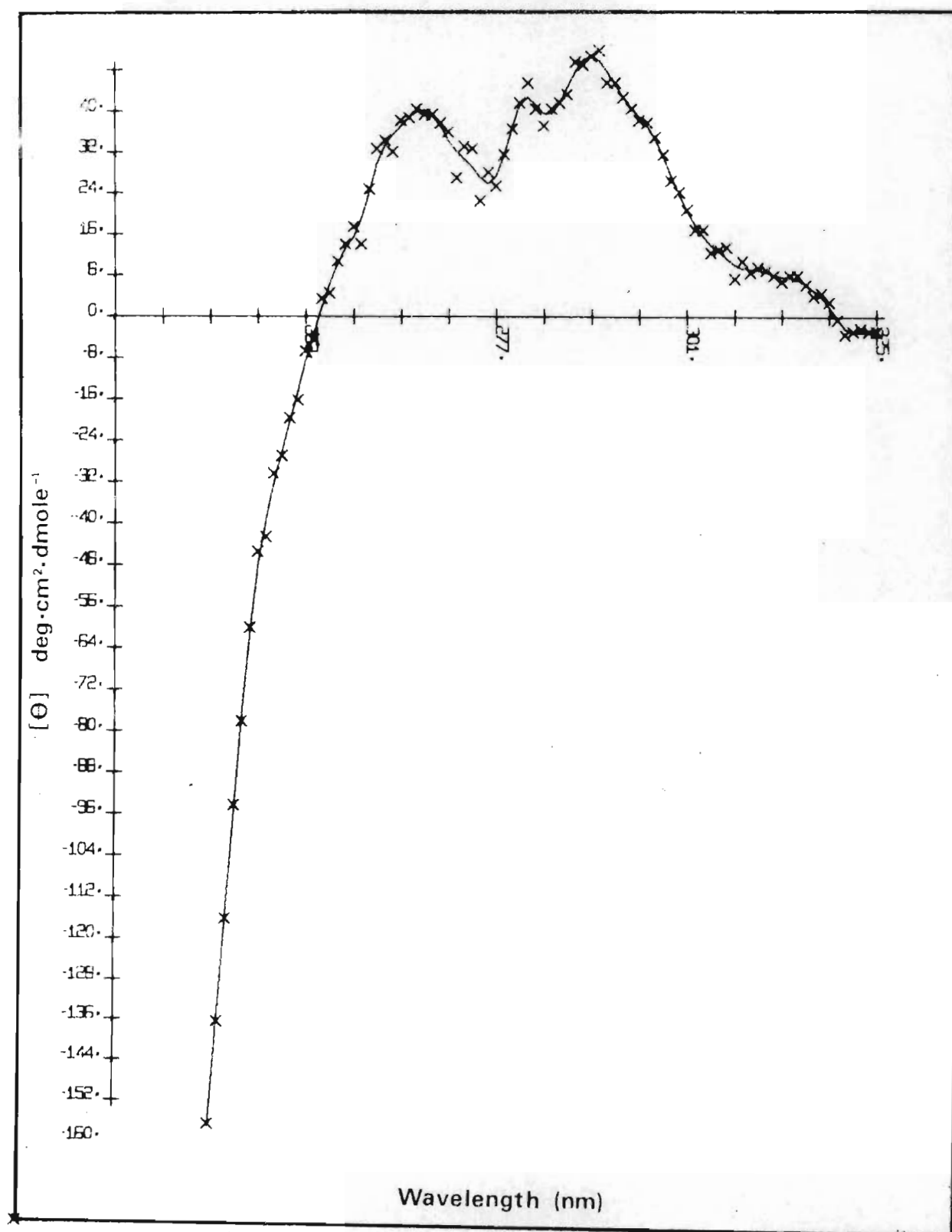


Fig. 34: CD spectrum (240-325 nm) of Fab μ (Sad) (fraction 21a) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.770 mg/ml)

have been observed in the case of normal L-chain preparations (Berggard and Peterson, 1969) and monoclonal Bence-Jones proteins (Bernier and Putnam, 1965; Gally and Edelman, 1964). The importance of this finding is that the presence and absence respectively of the ellipticity band at 235-236 nm in the spectra of fractions 17a and 17c may perhaps be due to different contents of these two L-chain conformations.

A negative ellipticity band at 243-245 nm was observed for the normal Fab μ fragments. Cathou et al. (1968) ascribe this band to the presence of random chain regions in the protein structure. Evidence supporting this hypothesis was found when exposure of rabbit Fab μ to 4 M- and 6 M-GuHCl led to a progressive increase in the amplitude of this band. Furthermore, such a band is seen in the CD spectra of randomly coiled poly-L-glutamate and poly-L-lysine (Veluz and Legend, 1965) and was theoretically predicted by Carver et al., (1966). This negative ellipticity band is however not observed for normal Fc μ or Fc μ (Sad) and is in agreement with the results of Ghose (1971) for a monoclonal IgM Fc μ -fragment. It could be that this band indicates the presence of a more significant proportion of randomly coiled regions in the Fab-part than in the Fc-part of immunoglobulins.

Another distinctive difference was evident in the near-UV region (260-300 nm) of the CD spectra of the Fab μ -fragments. Whereas normal Fab μ (fraction 17a, Fig. 30) displays a single positive Cotton effect at 290 nm ($[\theta] = 50 \text{ deg. cm}^2 \cdot \text{dmole}^{-1}$), the spectra of the more acidic normal Fab μ species (fraction

17c, Fig. 32) as well as Fab μ (Sad) (fraction 19b, Fig. 34) have considerably more fine structure, with other positive CD bands discernible near 270 nm and 280 nm in addition to the one at 290 nm. The latter two Fab μ 's therefore again displayed optical properties similar to each other but different from that of fraction 17a as was the case for the 235 nm band. It would appear that all Fab μ -species (normal and monoclonal) have some asymmetric ionised tyrosine side chains which are responsible for a CD band at 290 nm, but that not all of them contain the optically active chromophores (most probably other tyrosines or tryptophans) that give rise to the observed Cotton effects near 235 nm, 270 nm and 280 nm. It is important to note that the latter CD bands have now been observed in Fab μ 's derived from normal IgM (present investigation) as well as monoclonal IgM's and IgG's (present investigation; Ashman et al., 1971; Ghose, 1971; Cathou et al., 1968; Litman et al., 1971b). One would therefore conclude that the Fab μ -fragments from normal and monoclonal immunoglobulins (IgM and IgG) have very similar optical properties and probably also very similar conformations. Hence it is not feasible to classify an immunoglobulin as normal or monoclonal, human or animal on the basis of the CD properties of its Fab μ -fragment.

5.4.3.2 Fc μ (Sad) subfragment (fraction 19c). This fragment resulted from the high temperature trypsin digestion of IgM(Sad) and was isolated by molecular exclusion chromatography on Sephadex G-100 (Fig. 19, chapter 3). The CD spectra

of this subfragment (Figs. 35 and 36) showed no ellipticity bands in the near ultraviolet wavelength region, 260-300 nm; a strong negative ellipticity band ($[\theta] = -11,600 \text{ deg. cm}^2 \text{ dmole}^{-1}$) was however centred at 202 nm instead of 217 nm (c.f., $\text{Fc}_{5\mu}(\text{Sad})$, Appendix 4-Fig. A13). These properties are typical for a random coil conformation (Holzwarth and Doty, 1965) and fraction 19c therefore appears to have no ordered structure after being split from $\text{Fc}_{5\mu}(\text{Sad})$.

It was deduced earlier that fraction 19c originated from the amino-terminal part of $\text{Fc}_{\mu}(\text{Sad})$ (section 5.4.3.1) mainly because of the relatively high content of half-cystine residues of this fraction. The conformational significance of intrachain disulphide bonds in immunoglobulin structure was shown by Litman et al., (1971b) in a CD study of normal human IgG and its enzymic and chemically (reduction) derived fragments and peptide chains. They found that only the carboxy-terminal half of the Fc_{γ} (disulphide bonds intact) did not yield the typical ellipticity band at 217 nm and speculated that this band may originate from a cross-domain interaction. This hypothesis, (considering only the 217 nm band) was however negated by the results of Björk et al., (1971) who found that the V- and C-region compact domains of λ -chain, although having other differences in their CD spectra, both had strong 217 nm ellipticity bands. Their conclusion that these two halves of λ -chain probably exist as independently folded, non-interacting regions, is also borne out by the recent X-ray diffraction results of Poljak et al., (1972) on a human myeloma Fab γ .

5.4.3.3 Irreversibility of GuHCl denaturation. Early in this investigation (chapter 2) it was observed that after pool A proteins had been exposed to 8 M-Urea and the urea subsequently removed by dialysis, normal IgM could be detected only very weakly with monospecific anti-IgM antiserum, whereas a strong precipitin band had been obtained prior to urea-denaturation. It was argued that the decrease in antigenicity was caused by incomplete renaturation of the high molecular weight IgM upon removal of the urea denaturant.

Supporting evidence of this is found in the CD spectra of IgM(Sad) before (Figs. 37 and 38) and after (Figs. 39 and 40) exposure to 4 M-GuHCl. Similar signs of irreversible denaturation, or at least of incomplete renaturation, may be found in the spectra of normal Fc_{5u} (Appendix 4, Fig. A9 - A12). For both proteins investigated, GuHCl was removed by extensive dialysis against 0.075 M-phosphate buffer, pH 7.0, prior to the determination of CD spectra.

The most significant changes in these spectra were a complete loss of positive ellipticity bands in the 250-300 nm region (Figs. 38 and 40) and a blue shift of the 217 nm ellipticity band after the proteins had been exposed to GuHCl (Figs. 37 and 39). The absence of ellipticity bands in the near-UV associated with asymmetric aromatic side chains after renaturation of the denatured proteins (Figs. 38 and 40) indicates that such residues were not restored to their former restrictive spatial positions and the blue shift of the 217 nm band is expected if the renaturation attempt did not remove

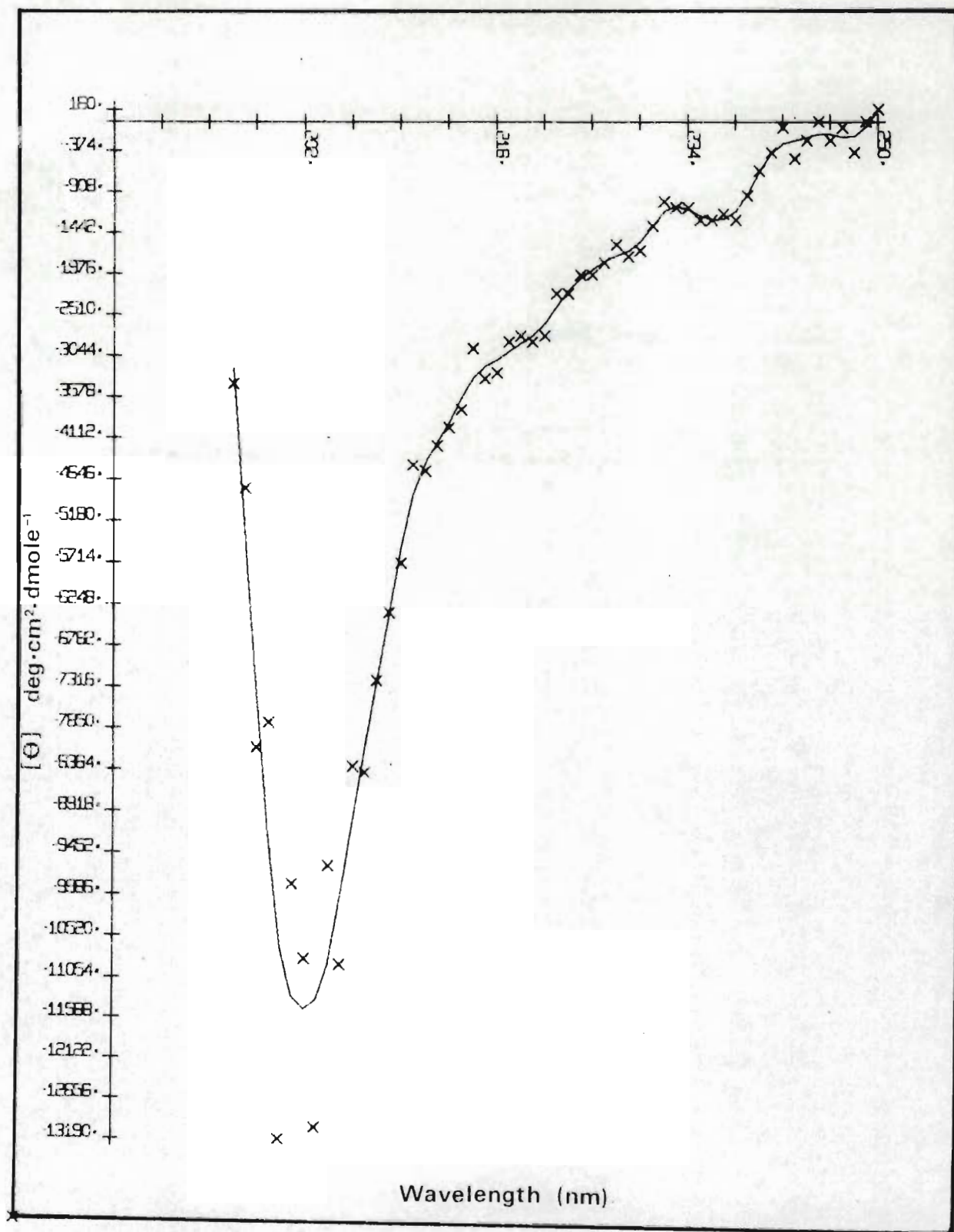


Fig. 35: CD spectrum (190-250 nm) of fraction 19c (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.272 mg/ml)

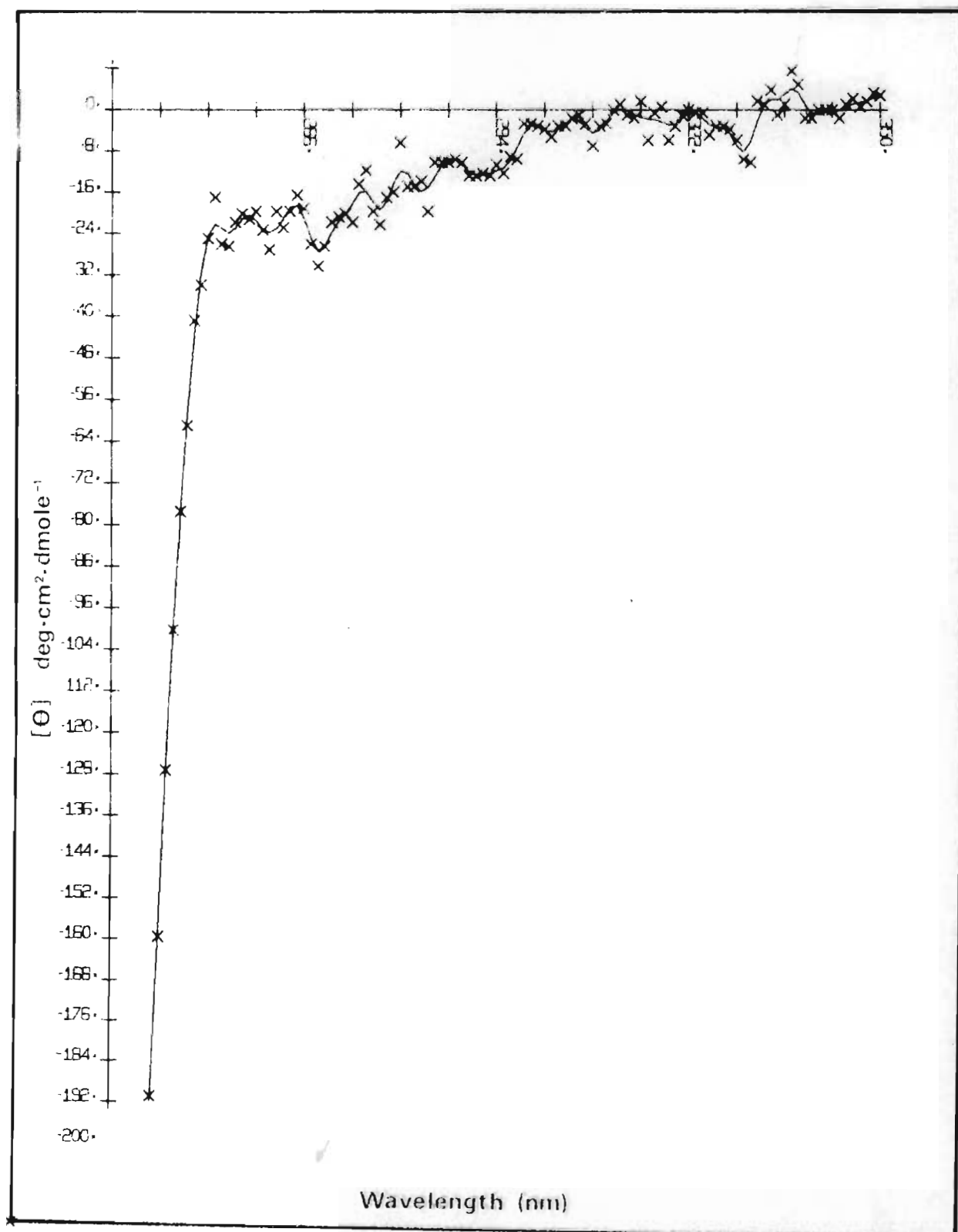


Fig. 36: CD spectrum (245-350 nm) of fraction 19c (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.815 mg/ml)

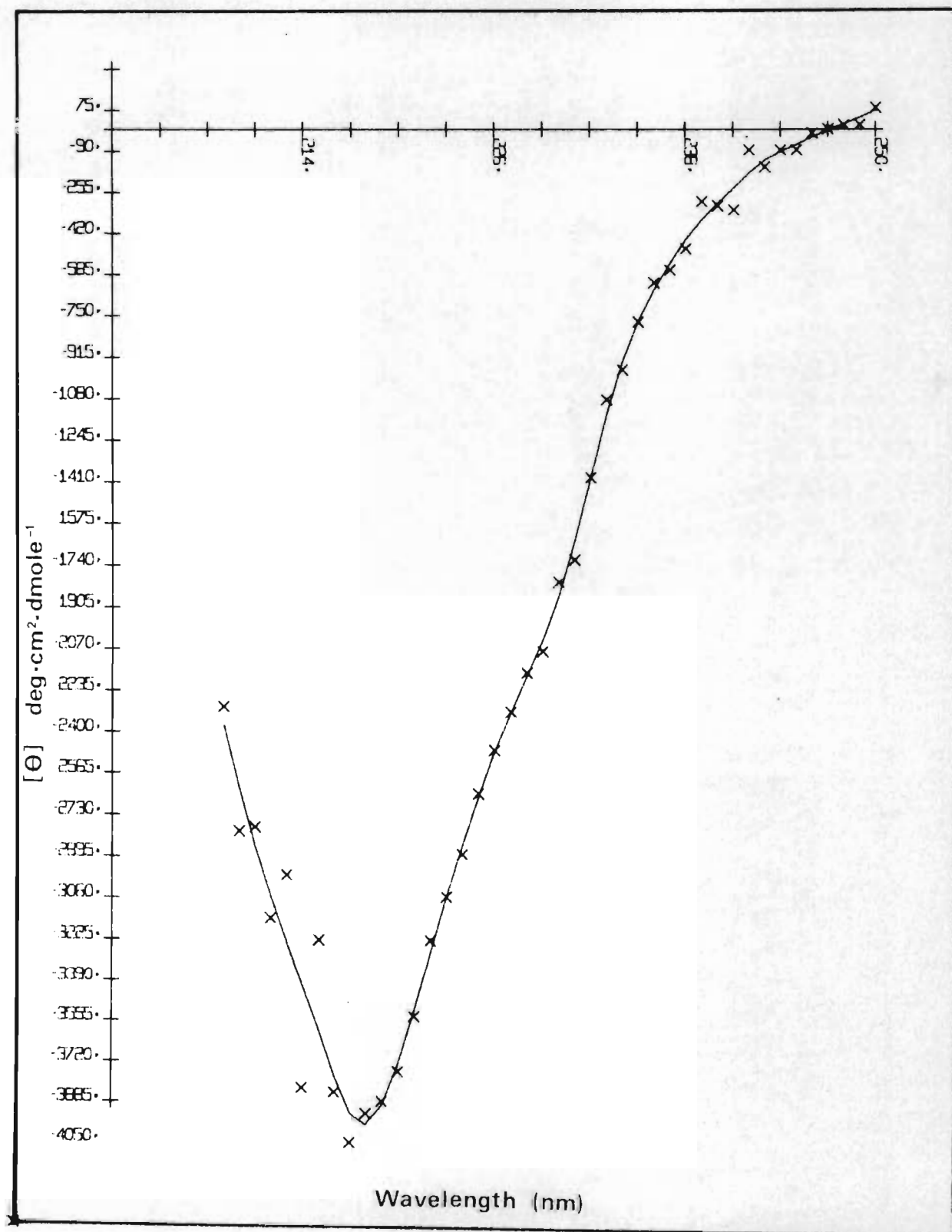


Fig. 37: CD spectrum (210-250 nm) of native IgM(Sad) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 1.260 mg/ml)

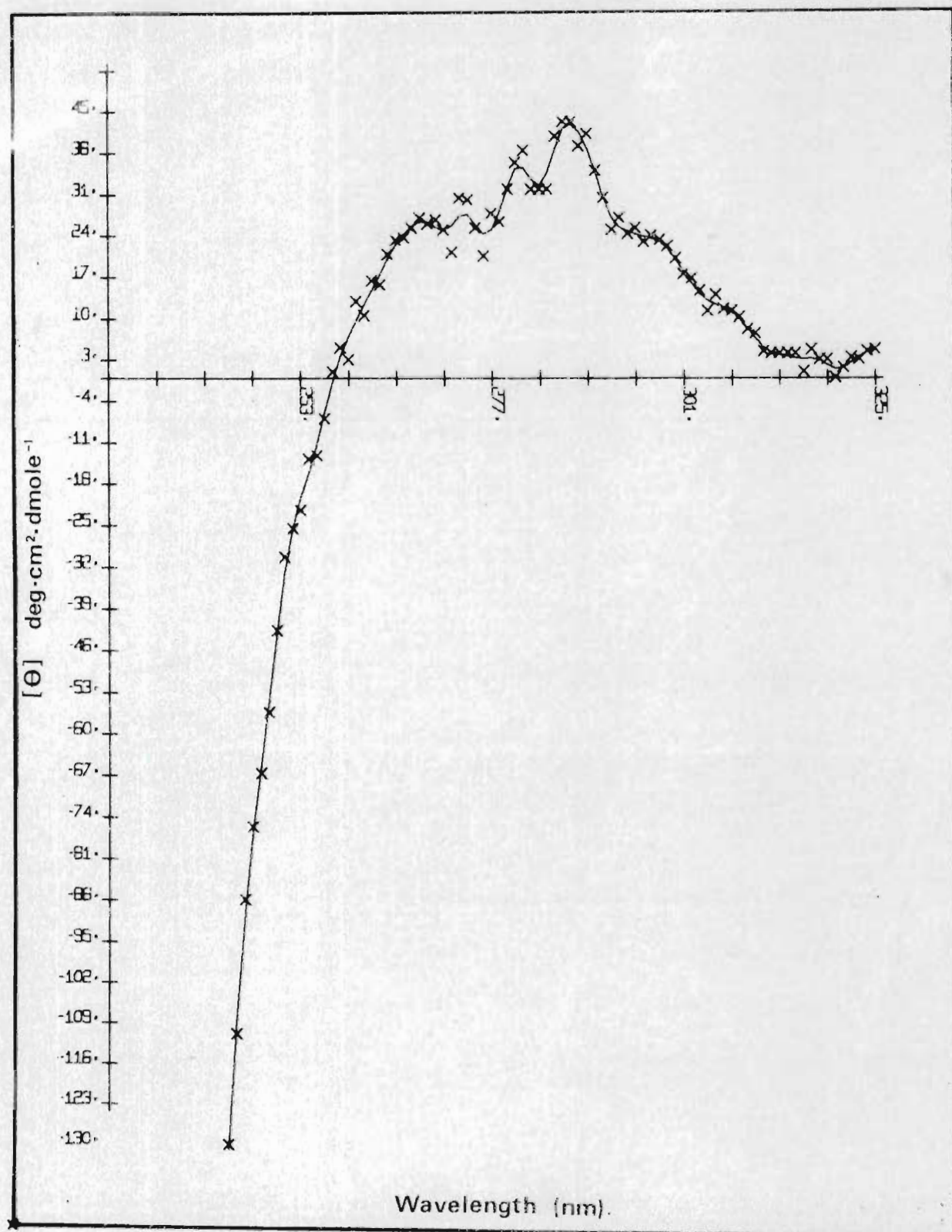


Fig. 38: CD spectrum (240-325 nm) of native IgM(Sad) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 1.260 mg/ml)

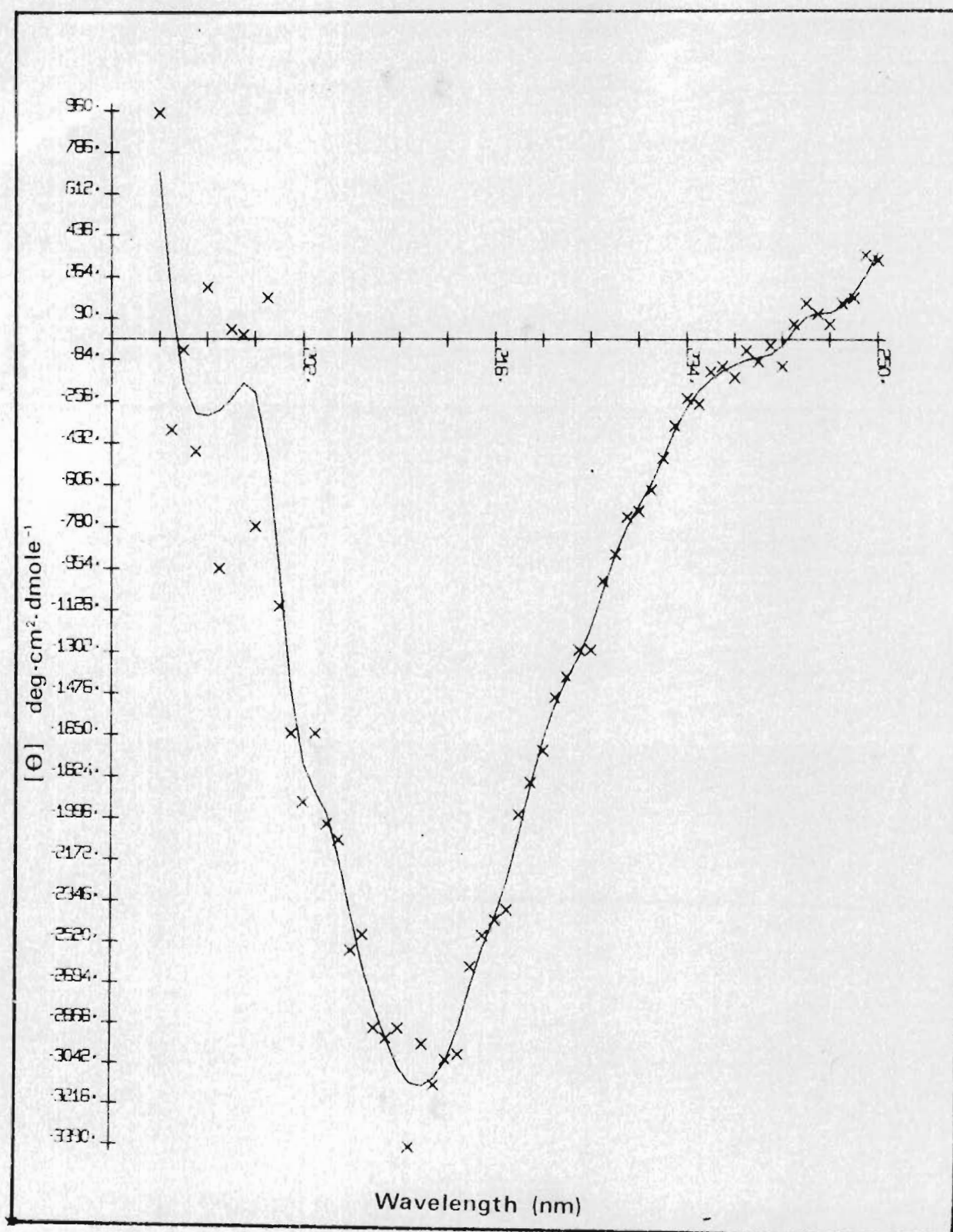


Fig. 39: CD spectrum (190-250 nm) of IgM(Sad) after renaturation from 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0 (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.195 mg/ml)

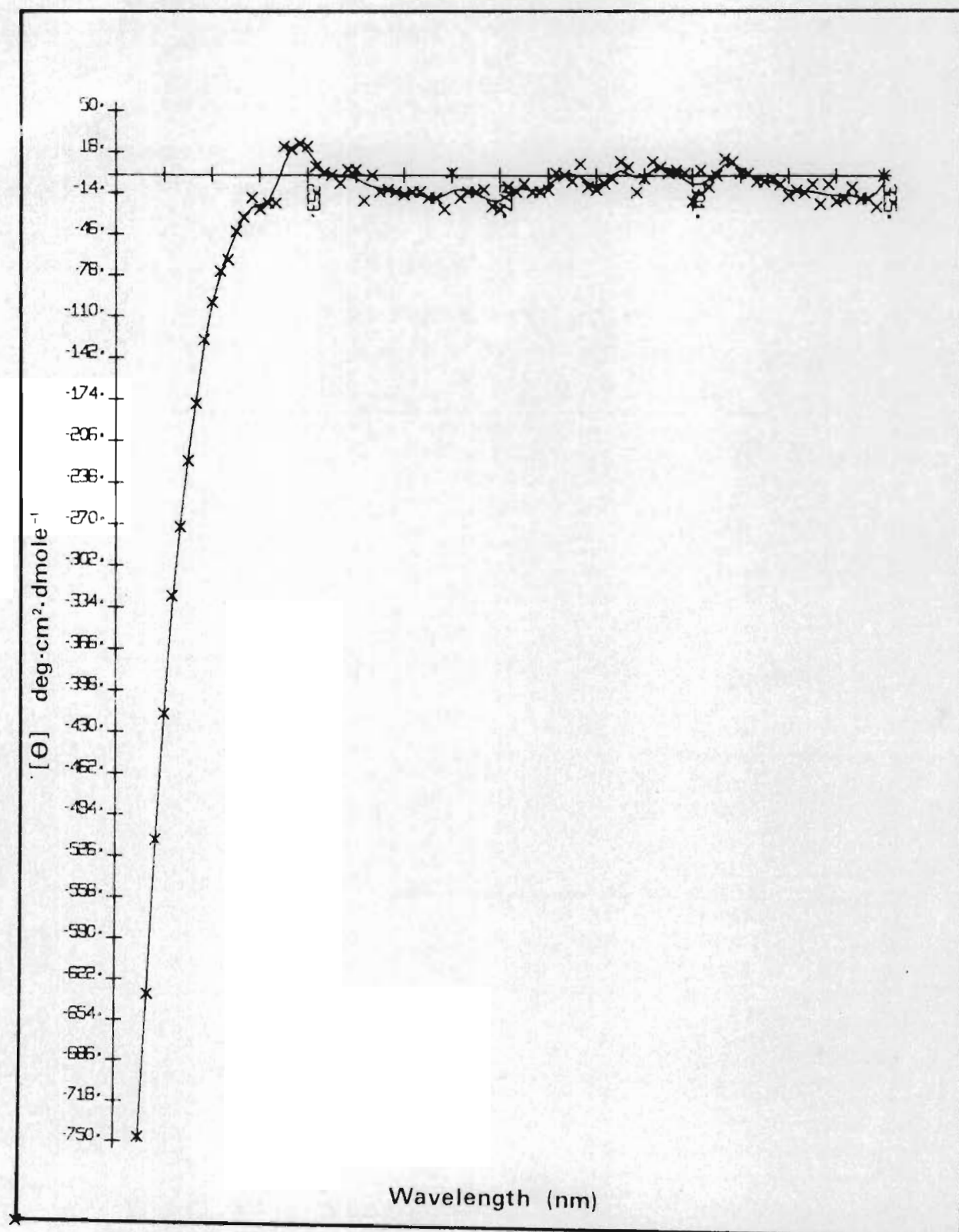


Fig. 40: CD spectrum (235-325 nm) of IgM(Sad) after renaturation from 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0 (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.195 mg/ml)

all random chain structure.

The renaturation of a protein dissolved in a denaturing solvent like 4 M-GuHCl not only depends on the rate of renaturation (i.e. removal of denaturant) but also on intrinsic characteristics like its content of hydrophobic and hydrophilic amino acid residues and in some cases apparently also on its size (Holt and Creeth, 1972). Several other suggestions have been put forward to explain incomplete renaturation (e.g. Ullman and Monod, 1969; Teipel and Koshland, 1971). All of these have one common feature, namely the involvement of a specific pathway, such as interaction with ligands or primitive folding of newly biosynthesised polypeptide chain, in order to bring about the eventual correct conformation of the protein.

The length of a polypeptide chain will also determine the number of thermo-dynamically acceptable stable states (i.e. conditions of minimum free energy) in which it can exist, and it is not surprising that most successful renaturation experiments were carried out on proteins of molecular weight under 20,000 daltons (Holt and Creeth, 1972). L-chain (M.W. approximately 23,000 daltons) is the smallest polypeptide component of the immunoglobulin proteins; it is therefore to be expected, on account of the foregoing considerations, that monomeric (IgG) and polymeric (IgA, IgM) immunoglobulins may not easily recover their correct or natural conformations after having been completely randomised. Furthermore, all immunoglobulins are known to be glycoproteins and it is conceivable that the hydrophilic

carbohydrate moieties may also adversely influence renaturation (Holt and Creeth, 1972).

5.5 CONCLUSION

Some indication of the comparable nature of normal IgM, IgM(Sad) and their fragments was obtained earlier from their SDS-PAGE and IE analyses (chapters 2, 3 and 4). Sedimentation coefficient and molecular weight determinations on normal IgM, IgM(Sad) and their derivatives showed that, except for IgM_s(Sad), all other proteins examined gave results which were in good agreement with previously reported values obtained for monoclonal IgM's and their subfragments and constituent chains. The low molecular weight of IgM_s(Sad) and the too high molecular weight of IgM(Sad) were examined and discussed on the basis of the 10 μ + 15L-chain model proposed by Suzuki and Deutsch (1967). The most important deduction to be made from the molecular weight estimates was that normal IgM and IgM(Sad) both conformed to the pentameric structure accepted for this class of immunoglobulin.

The CD spectra of some normal IgM and IgM(Sad) fragments and of the parent molecules showed that the monoclonal and polyclonal IgM investigated have similar overall conformations in solution. The differences in biological properties of immunoglobulins are therefore not expressed at a gross conformational level, but can be expected to be manifest at a more local level of structural organisation. This cannot be readily examined with the aid of a technique such as CD spectroscopy.

Chapter 6

GENERAL DISCUSSION AND CONCLUSION

The primary objective of the investigations reported in this thesis was to establish whether or not monoclonal Waldenström IgM, from which stems almost our entire knowledge regarding the structure and conformation of this immunoglobulin class, is a valid model on which to base deductions and predictions about normal IgM and its role in immunology. In order to ascertain this a study was indicated in which physico-chemical comparisons could be made between normal and monoclonal IgM.

To this end a method was developed by means of which sufficient quantities of normal IgM could be isolated from a waste product of plasma fractionation. This method entailed the extraction of IgM from Cohn fraction III and the subsequent isolation, by means of molecular exclusion chromatography, of a high molecular weight fraction containing most of the IgM. Final purification was achieved through the use of an immunoabsorbent developed specifically against contaminants in the partially purified IgM preparation. The nature of the proteins removed by immunoabsorption was not fully determined but immunodiffusion analysis of these "contaminants" indicated the presence of naturally-occurring macroglobulins as well as proteins with a molecular weight which, under normal conditions, should have precluded their appearance at the same elution volume as that of IgM. These lower molecular weight proteins included IgG and C3 and their presence suggested, because of their known capacity for interaction, e.g. during cytolysis, that they were present as large soluble complexes. The complex nature of the IgG in this high M.W. fraction was later verified by the finding

that the Fc-part of these molecules was masked to such an extent that they could not be completely removed with the aid of insolubilised anti-Fc γ antiserum. Moreover, it was found that an anti-Fd γ immunoadsorbent was also incapable of removing all IgG contamination, indicating that its antigen binding part (i.e. Fd γ) was therefore also involved in complex formation. These observations served to explain the early, perplexing findings that separation methods based on molecular size or charge differences of proteins were inadequate when applied to the purification of IgM extracted from Cohn fraction III.

To ascertain whether the "architecture" of monoclonal and polyclonal IgM is the same or at least comparable, normal IgM and a Waldenström IgM were subjected to high temperature tryptic proteolysis and the resultant fragments were separated by molecular exclusion chromatography. The molecular size heterogeneity of these presumably monodisperse fragments was unexpected, but at the same time it afforded an indication of the inadequacy of methods like molecular exclusion chromatography and immunoelectrophoresis as sole tests for homogeneity. It would appear that unless so-called "pure" immunoglobulin fragments are also subjected to analysis by polyacrylamide gel electrophoresis in the presence of a dissociating agent like sodium dodecyl sulphate, their molecular size homogeneity remains suspect.

Another unexpected observation was that the presence of NaCl in digestion mixtures had a marked effect on the rate of tryptic digestion of both normal IgM and IgM(Sad) and it was concluded that this was due to some form of structural stabilization of IgM by NaCl. This observation perhaps deserves to be more fully investigated; experiments

in which IgM is subjected to trypsinolysis in the presence of various concentrations of neutral salts of a Hofmeister series could perhaps reveal optimum conditions under which to secure either $Fab\mu$ or $Fc_{5\mu}$, or both.

In order to obtain fragments smaller than the proteolytic ones, the latter were reduced and alkylated in standard fashion. This chemical method of dissociation was also employed to obtain the μ -chains of normal and monoclonal IgM in which reside the intrinsic differences determining the various immunoglobulin classes. These component fragments and peptide chains as well as their respective precursor molecules were examined by means of ultracentrifugation (sedimentation coefficients and molecular weight determinations) and circular dichroism spectroscopy. From the physico-chemical parameters determined with the aid of these two techniques and also from observations made on the column chromatographic, immunoelectrophoretic and SDS-PAGE behaviour of comparable components of normal IgM and IgM(Sad), it was concluded that normal IgM behaves like its monoclonal counterpart in most respects, but some disparities were also observed.

Some of the incongruities noted were the different susceptibilities of the intact molecules to tryptic fragmentation ($IgM(Sad) > normal\ IgM$); an apparent easier further proteolytic breakdown of $Fab\mu(Sad)$; the formation from IgM(Sad) of a homogeneous tryptic fragment with a lower molecular weight than that of L-chain and a significant difference in the molecular weights of the intact normal and monoclonal IgM molecules. This last-mentioned difference is perplexing and difficult to relate to deductions made from summations of the molecular weights of component proteolytic fragments or H- and L-chains.

CD spectroscopy proved to be a useful probe of the conformations in solution of the immunoglobulins and of their fragments. From the CD spectra it was concluded that, except for some minor differences, all comparable normal and monoclonal species had very similar conformations. Although attempts were made to detect a change in the conformation of IgM at high temperatures (56°C) in order to explain the better yield of $\text{Fc}_5\mu$ relative to trypsin digests at a lower temperature (37°C), these were unsuccessful. More conclusive results in this regard could perhaps be obtained if a chromophore with an absorption band in the visible region were first attached to the molecule, preferably near the hinge area. Such an IgM derivative may possibly display new intrinsic ellipticity bands in the visible region which, being outside the ultraviolet region where the protein itself has intrinsic Cotton effects, would be more sensitive indicators of small temperature-dependent changes in the conformation of IgM.

Some problems regarding the structure of IgM still await solution. One of these is the role of J-chain (joining chain) discovered recently in IgA (Halpern and Koshland, 1970) and subsequently detected in IgM (Mestecky *et al.*, 1971) and in the macroglobulins of the pheasant, marine toad, catfish (Weinheimer *et al.*, 1971) and mouse (Parkhouse, 1972). This polypeptide has been shown to be quite distinct from α -, μ - and L-chain (Morrison and Koshland, 1972) and its proposed joining function in the polymeric immunoglobulins was confirmed by Parkhouse (1972), who found that it is not bound to IgM_5 prior to secretion, but occurs covalently linked to extracellular IgM. Further support has also come from quantitative determination on the stoichiometry and number of half-cystine residues involved in the covalent binding of J-chain to polymeric IgA and IgM (Morrison and Koshland, 1972; Mestecky *et al.*, 1972). Although

Parkhouse et al. (1970) found a very specific and high degree of re-association of reduced but non-alkylated IgM on reoxidation, it is difficult to envisage a globular J-chain of molecular weight 24,000 daltons (Morrison and Koshland, 1972) spanning the 10 bulky $F_{c\mu}$ -regions which are covalently linked in IgM, and at the same time having the appropriate half-cystine residues in correct stereochemical juxtaposition for crosslinking it to the $F_{c\mu}$ -regions. However, the J-chain could be envisaged to exist as an extended polypeptide, allowing it to form a "circular bridge" between individual subunits. Thus, in 19-S IgM, the F_c -regions of individual IgM subunits may not be directly linked, but rather via a single J-chain with half-cystine residues spaced at regular intervals along its length. Calculation of the maximum length of a fully extended J-chain ($\frac{24,500}{108(M.R.W.)} \times 3.63 \overset{O}{\text{\AA}} - 823 \overset{O}{\text{\AA}}$) shows that such a polypeptide could easily span the circumference of a circle through even the hinge region of IgM ($2 \pi \times 105 \overset{O}{\text{\AA}} = 660 \overset{O}{\text{\AA}}$; Metzger, 1970). If an extended circular structure does exist for J-chain in the native molecule, CD spectroscopy of this protein, isolated under milder conditions, could perhaps detect such a structure. Information regarding the possible role of J-chain in the in vitro re-association of reduced, non-alkylated IgM_5 may also be obtained, if J-chain were to be removed by immunoadsorption with an anti-J-chain immunoadsorbent from IgM which has only been reduced. Comparison by means of SDS-PAGE of the re-association products or determination of the association constants for this reaction in the presence and absence of J-chain, could shed more light on the actual importance of this molecule in the structure of polymeric immunoglobulins.

As previously stated, most structural and conformational analysis has been conducted on Waldenström IgM's, the reasons for this being two-fold, namely the homogeneity of the monoclonal IgM species and the relative abundance of this material in macroglobulinaemic plasma. The work reported in this thesis has indicated that there are many similarities between normal IgM and a specific Waldenström IgM (IgM(Sad)) isolated in the author's laboratory, but some disparities have also been noted. That there are such disparities should not be regarded as totally surprising, because in the case of the Waldenström macroglobulins one is studying a single IgM species which might have some unique properties conferred on it by its unique V-region. Such a species might well exist in the normal IgM pool, but in a concentration too low to permit its detection. The present study has provided substantial evidence that, at least as regards gross conformation and structure, Waldenström IgM may justifiably be used as a model of normal IgM, and that information derived from a study of monoclonal IgM might be extrapolated to normal IgM with a greater degree of confidence.

S U M M A R Y

- 1 Normal IgM was extracted and purified from Cohn fraction III of pooled normal human plasma using the following procedures:
 - (a) extraction with 0.1 M acetate buffer, pH 4.1, in a ratio of 500 g Cohn fraction III : 2 L buffer;
 - (b) clarification by centrifugation at 35,500 xg for 1 h at 4°C.
 - (c) precipitation of macroglobulins either by euglobulin precipitation (low ionic strength) or 8% PEG 4,000;
 - (d) delipidation by ultracentrifugal flotation after the density of the IgM-containing solution had been increased to 1.2 g/ml with NaBr;
 - (e) chromatography on 3.5% agar beads to remove aggregated material and low molecular weight components;
 - (f) removal of non-IgM proteins by immunoabsorption.
- 2 Monoclonal IgM(Sad) was purified by:
 - (a) precipitation from macroglobulinaemic plasma with 7% PEG 4,000;
 - (b) column chromatography on 3.5% agar beads.
- 3 Purified normal IgM and IgM(Sad) were digested with trypsin (56°C) for various times and in the presence of different NaCl concentrations. Fragmentation products were analysed by means of SDS-PAGE and IE and it was found that NaCl suppressed trypsin digestion.
- 4 $Fc_{5\mu}$ and Fab_{μ} fragments were isolated from large scale high temperature trypsin digests of normal IgM and IgM(Sad) by

means of column chromatography in the presence of 4.0 M-GuHCl after the Fab μ had been chromatographed on DEAE-cellulose ion exchange resin.

- 5 A fast anodal component was detected (IE) and separated from the trypsin digest of normal IgM and a low molecular weight subfragment of Fc μ (Sad) was isolated from the trypsin digest of IgM(Sad).
- 6 Monomeric normal Fc μ and Fc μ (Sad) were obtained by reduction and alkylation of the respective pentameric precursors, and were purified by molecular exclusion chromatography in 4.0 M-GuHCl.
- 7 The μ - and L-chains of normal IgM and IgM(Sad), reduced and alkylated in the presence of 4.0 M-GuHCl, were isolated by column chromatography on Sephadex G-200.
- 8 The sedimentation coefficients, molecular weights and CD spectra of normal IgM, IgM(Sad) and some of their tryptic fragments and reduced and alkylated subunits and polypeptides were determined.
- 9 The M.W. values of the tryptic fragments and the μ - and L-chains of normal IgM indicated that this molecule conformed to the generally accepted 10 μ + 10 L-chain model for this immunoglobulin class....
- 10 Although M.W. values for Fc μ (Sad) and monomeric Fc μ (Sad) proved that IgM(Sad) was also a pentameric molecule, the discrepancy between the M.W.'s of the intact molecule and its component μ - and L-chains precluded the unequivocal deduction of a 10 μ + 10 L-chain model for this immunoglobulin.

- 11 The CD spectra of normal IgM, IgM(Sad) and their tryptic fragments and reduced and alkylated subunits and polypeptide chains indicated that they have similar conformations.
- 12 The irreversible loss of conformation of IgM(Sad) on denaturation by GuHCl was shown by the dissimilarity in CD spectra of this molecule before and after exposure to 4.0 M-GuHCl.
- 13 It was concluded that normal IgM conformed in most instances to the parameters determined for IgM(Sad) and other monoclonal IgM's reported in the literature.

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APPENDIX 1

Computer programme used for the calculation of sedimentation coefficients ($S_{20,w}^0$) and examples of input and printout data sheets.

```

C---- RUN ON AN I.B.M. 1130 COMPUTER WITH 8K CORE.
C---- 1132 LINE PRINTER AND 1442 CARD READER.
C---- COMPUTER CENTRE - UNIVERSITY OF NATAL.
C CALCULATION OF SEDEMENTATION COEFFICIENTS
C J. CONRADIE - N.B.T.S.
  DIMENSION TIME(50),Y(50),CONC(20),SW(20),NAME(20),CS(50)
  PI=3.14159265
  READ(2,100) NEXP,NAME
100  FORMAT(12,2X,20A2)
C---- NEXP - NUMBER OF EXPERIMENTS
C---- N - NUMBER OF OBSERVATIONS.
C---- RPM - REVOLUTIONS PER MINUTE.
C---- FACT - CORRECTION FACTOR.
C---- CONS - CONCENTRATION.
  DO 10 JEXP=1,NEXP
  READ(2,101) N,RPM,FACT,CONS
101  FORMAT(12,F8.0,F8.4,F6.1)
  DO 2 J=1,N
  READ(2,102) TIME(J),CS(J)
102  FORMAT(F10.0,F10.2)
C---- TIME - TIME IN MINUTES.
C---- CS - RADIAL DISTANCE IN CMS.
  Y(J)=ALOG(CS(J))
  2  CONTINUE
  CALL FIT(N,TIME,Y,A,B,SER)
C---- THIS FITS THE STRAIGHT LINE Y=A+B*TIME
  W=RPM/60.*(2.*PI)
C---- W - OMEGA ROTATION IN RADIANS PER SECOND.
  WRITE(3,103) N,RPM,W,FACT,CONS
103  FORMAT(1H1,12,' OBSERVATIONS R.P.M.=',F7.0,5X,'W=',F10.1,5X,
1  'FACT=',F8.4,5X,'CONCENTRATION=',F5.1/)
  WRITE(3,104)
104  FORMAT(7X,'TIME',7X,'CMS',6X,'LOG',7X,'EST',6X,'DIFF'/)
  DO 3 J=1,N
  YEST=A+B*TIME(J)
  DIFF=Y(J)-YEST
  WRITE(3,105) TIME(J),CS(J),Y(J),YEST,DIFF
105  FORMAT(1X,F10.0,F10.2,F10.4,F10.4,F10.4)
  3  CONTINUE
C---- SOBS - OBSERVED SEDEMENTATION CO-EFFICIENT.
  SOBS=B/(60.*W*W)*1.E13
C---- S20W - CORRECTED SEDEMENTATION CO-EFFICIENT AT 20 DEG.
  S20W=FACT*SOBS
C---- COMPUTED VALUES SAVED FOR SUBSEQUENT REGRESSION.
  SW(JEXP)=S20W
  CONC(JEXP)=CONS
  WRITE(3,106) B,SOBS,S20W,SER
106  FORMAT(1H0,'SLOPE=',F8.5,5X,'SOBS=',F6.2,5X,'S20W=',F6.2,5X,
1  'STD.ERROR=',F8.5)
10  CONTINUE

```

```

      CALL FIT(NEXP,CONC,SW,A,B,SER)
C---- THIS FITS THE STRAIGHT LINE SW=A+B*CONC
C---- TO ENABLE US TO FIND INTERCEPT AT ZERO.
      WRITE(3,107) NAME
107   FORMAT(1H1,10X,20A2/)
      WRITE(3,108)
108   FORMAT(7X,'CONC',6X,'S20W',7X,'EST',6X,'DIFF'/)
      DO 4 J=1,NEXP
      YEST=A+B*CONC(J)
      DIFF=SW(J)-YEST
      WRITE(3,109) CONC(J),SW(J),YEST,DIFF
109   FORMAT(1X,F10.2,F10.2,F10.2,F10.2)
4     CONTINUE
      WRITE(3,110) A,B,SER
110   FORMAT('0','S20W AT CONC=0',F7.3,5X,'SLOPE=',F8.4,5X,'STD.ERROR='
1      F8.4)
C---- THE PROCEDURE IS REPEATED WITH THE RECIPROCAL.
      DO 5 J = 1,NEXP
      SW(J) = 1./ SW(J)
5     CONTINUE
      CALL FIT(NEXP,CONC,SW,A,B,SER)
      WRITE(3,111)
111   FORMAT(1H0,6X,'CONC',5X,'RECIP',7X,'EST',6X,'DIFF'/)
      DO 6 J = 1,NEXP
      YEST = A + B * CONC(J)
      DIFF = SW(J) - YEST
      WRITE(3,112) CONC(J),SW(J), YEST,DIFF
112   FORMAT(1X,F10.2,3F10.6)
6     CONTINUE
      SREC = 1. / A
      WRITE(3,110) SREC,B,SER
      STOP
      END

```



```

C----- RUN ON AN I.B.M. 1130 COMPUTER WITH 8K CORE.
C----- COMPUTER CENTRE - UNIVERSITY OF NATAL.
SUBROUTINE FIT(N,X,Y,A,B,SIGYX)
C----- THIS SUBROUTINE FITS THE STRAIGHT LINE  $Y=A+B*X$ 
DIMENSION X(50),Y(50)
SX=0.
SY=0.
SXX=0.
SXY=0.
SYY=0.
DO 2 J=1,N
SX=SX+X(J)
SY=SY+Y(J)
SXX=SXX+X(J)**2
SYY=SYY+Y(J)**2
SXY=SXY+X(J)*Y(J)
2 CONTINUE
RN=FLOAT(N)
XM=SX/RN
YM=SY/RN
DX2=RN*SXX-SX*SX
DY2=RN*SYY-SY*SY
DXY=RN*SXY-SX*SY
B=DXY/DX2
A=YM-XM*B
SIGYX=SQRT(DY2-B**2*DX2)/(RN-1.)
RETURN
END

```


10 OBSERVATIONS	R.P.M.= 56821.	W=	5950.2	
FACT= 1.0998	CONCENTRATION=	4.1		
TIME	CMS	LOG	EST	DIFF
0.	12.54	2.5294	2.5292	0.0001
4.	12.61	2.5345	2.5346	-0.0000
8.	12.68	2.5401	2.5400	0.0000
12.	12.74	2.5453	2.5454	-0.0001
16.	12.81	2.5509	2.5509	0.0000
20.	12.88	2.5562	2.5563	-0.0001
24.	12.95	2.5616	2.5617	-0.0001
28.	13.02	2.5671	2.5671	-0.0000
32.	13.10	2.5727	2.5726	0.0001
36.	13.17	2.5781	2.5780	0.0001

SLOPE= 0.00135 SOBS= 6.38 S2OW= 7.02
STD.ERROR= 0.00227

75 IGN SAD 136B

CONC	S20W	EST	DIFF
6.20	7.39	7.16	0.22
5.20	6.94	7.15	-0.21
4.17	7.02	7.14	-0.12
3.10	7.11	7.13	-0.01
2.08	7.24	7.12	0.12

S20W AT CONC=0 7.102 SLOPE= 0.0100
 STD.ERROR= 0.1996

CONC	RECIP	EST	DIFF
6.20	0.135278	0.139709	-0.004431
5.20	0.144035	0.139869	0.004165
4.17	0.142383	0.140033	0.002349
3.10	0.140544	0.140204	0.000340
2.08	0.137943	0.140367	-0.002424

S20W AT CONC=0 7.107 SLOPE= -0.0001
 STD.ERROR= 0.0038

APPENDIX 2

Computer programme used for the calculation of molecular weights
and examples of input and printout data sheets.

```

C----- RUN ON AN I.B.M. 1130 COMPUTER WITH 8K CORE.
C----- 1132 LINE PRINTER AND 1442 CARD READER.
C----- COMPUTER CENTRE - UNIVERSITY OF NATAL.
C..... CALCULATION OF MOLECULAR WEIGHTS - J.CONRADIE (N.B.T.S.)
          DIMENSION R(50),DO(50),R2(50),Y(50),NAME(20)
          DIMENSION NNRUN(20),WTMS(20),SERS(20)
C          R - MEASURED R
C          DO - OPTICAL DENSITY
C          R2 - ( R/MF + 5.7 ) ** 2
C          Y - LN(OD)
C          NAME - NAME OF EXPERIMENT - 40 CHARACTERS
          KOUT=3
          KIN=2
          CR=8.313E07
          TOPI=2.*3.14159265
C----- NN- NUMBER OF EXPERIMENTAL RUNS.
          READ(KIN,119)NN
119      FORMAT(12)
          DO 99 JN=1,NN
          READ(KIN,100)NCOMP,NRUN,N,NAME
100      FORMAT(3I4,20A2)
          READ(KIN,101)FM,T,RPM,DEN
101      FORMAT(4F12.4)
C----- FM - MAGNIFICATION FACTOR. TO BE DIVIDED BY 1.6
C----- T - ABSOLUTE TEMPERATURE.
C----- RPM - REVOLUTIONS PER MINUTE.
C----- DEN - BOUYANCY CONSTANT.
          WW=(RPM/60.*TOPI)**2
C----- WW - OMEGA SQUARED.
          FMM=FM/1.6
          READ(KIN,106)(R(J),DO(J),J=1,N)
106      FORMAT(2F8.5)
          DO 4 J=1,N
          R2(J)=(R(J)/FMM+5.7)**2
          Y(J)=ALOG(DO(J))
4          CONTINUE
C
C          LINEAR REGRESSION OF Y ON R**2
          CALL FIT(N,R2,Y,A,B,SER)
C
          WRITE(KOUT,113) NCOMP,NRUN
113      FORMAT(1H1,'COMPOUND',14,5X,'RUN NO.',14)
          WRITE(KOUT,102) NAME,FM,T,WW,DEN
102      FORMAT(1X,20A2,2X,'MAGNIFICATION FACTOR =',F6.2,'/1.6',4X,
*          'TEMP.=' ,F6.2/43X,'W2=' ,E12.5,4X,'DENOM=' ,F8.5)

```



```

WRITE(KOUT,103)
103  FORMAT(6X,'R',6X,'R**2',6X,'O.D.',4X,'LN(OD)',7X,'EST',6X,'DIFF
      DO 6 J=1,N
      YE=A+B*R2(J)
      DIFF=Y(J)-YE
      WRITE(KOUT,104) R(J),R2(J),DO(J),Y(J),YE,DIFF
104  FORMAT(1X,F8.2,F8.2,F10.5,F10.5,F10.5,F10.5)
      6 CONTINUE
C---- B - SLOPE OF THE GRAPH USING NATURAL LOGS.
      SLOPE=B/ALOG(10.)
C---- SLOPE - SLOPE OF THE GRAPH USING LOGS TO THE BASE 10.
      F1=CR*T/(DEN*WW)
      WTM=2.*F1*B
C---- WTM - MOLECULAR WEIGHT.
      WRITE(KOUT,105) F1,WTM,SER,SLOPE
105  FORMAT(1H0,'F=',F10.2,5X,'MOL WT.=',F10.2,5X,'STD. ERROR',F8.5,
      1      5X,'SLOPE=',F8.5)
C
C---- COMPUTED VALUES SAVED FOR SUMMARY AT THE END.
C
      WTMS(JN)=WTM
      SERS(JN)=SER
      NNRUN(JN)=NRUN
99   CONTINUE
C---- SUMMARY OF CALCULATIONS.
      SUM=0.
      SS=0.
      WRITE(KOUT,112)
112  FORMAT('1SUMMARY'//1X,' COMP RUN MOL WT ST ERR')
      DO 7 K=1,NN
      SUM=SUM+WTMS(K)
      SS=SS+WTMS(K)**2
      WRITE(KOUT,110)NNRUN(K),WTMS(K),SERS(K)
110  FORMAT(7X,16,F10.1,F10.4)
      7 CONTINUE
      AVE=SUM/FLOAT(NN)
      SIG=SQRT((SS-SUM*AVE)/FLOAT(NN))
      WRITE(KOUT,111)AVE,SIG
111  FORMAT(1H0,'AVE=',F10.2,10X,'STD.DEV.=',F8.2)
      STOP
      END

```

Cols

Compound Ref. No.
Experimental Run No.
No. of Observations

		1	5
			1
		1	6

1 - 4
5 - 8
9 - 12

N	O	r	m	a	i	F	c	u	2	2	0	6				
b	M		G	u	H	C	I									

13 - 52

$$(1 - \nu \rho)$$

							2	7	.	5	
						2	9	2	.	8	5
						3	3	0	8	7	.
						.	1	9	1	7	

1 - 12
13 - 24
25 - 36
37 - 48

[illegible]

COMPOUND 15 RUN NO. 1 MAGNIFICATION FACTOR = 27.50/1.62
 NORMAL FCU 220B 6M GUHCL W2= 0.12005E 08 DENOM= 0.19170
 TEMP.=292.85

R	R**2	O.D.	LN(OD)	EST	DIFF
4.90	35.86	0.100	-2.30258	-2.28763	-0.01495
5.00	35.93	0.115	-2.16282	-2.16793	0.00511
5.10	36.00	0.130	-2.04022	-2.04811	0.00789
5.20	36.07	0.147	-1.91732	-1.92817	0.01084
5.30	36.14	0.163	-1.81400	-1.80811	-0.00588
5.40	36.21	0.185	-1.68739	-1.68795	0.00055
5.50	36.28	0.207	-1.57503	-1.56766	-0.00737
5.60	36.35	0.235	-1.44816	-1.44724	-0.00092
5.70	36.43	0.260	-1.34707	-1.32672	-0.02034
5.80	36.50	0.300	-1.20397	-1.20608	0.00211
5.90	36.57	0.333	-1.09961	-1.08531	-0.01429
6.00	36.64	0.380	-0.96758	-0.96444	-0.00313
6.10	36.71	0.435	-0.83240	-0.84343	0.01102
6.20	36.78	0.485	-0.72360	-0.72231	-0.00129
6.30	36.85	0.555	-0.58878	-0.60108	0.01229
6.40	36.93	0.630	-0.46203	-0.47974	0.01770

F= 10578.13 MCL WT.= 35874.60 STD. ERROR 0.04530
 SLOPE= 0.73643

SUMMARY

COMP	RUN	MOL WT	ST ERR
	1	35874.6	0.0453
	2	36532.1	0.0693
	3	36532.9	0.0734
	4	35287.2	0.0418
	5	36043.7	0.0693
	6	34093.0	0.0810
	7	35322.6	0.0327
	8	34488.1	0.0575
	9	35212.8	0.0422
	10	36520.3	0.0324
	11	36210.5	0.0594
	12	35849.2	0.0717

AVE= 35663.94

STD.DEV.= 768.40

APPENDIX 3

Computer programme used for the calculation of mean residue ellipticities and plotting of CD spectra.

```

C
C***** INPUT *****
C
C----- TABLE OF HS VALUES
C----- TABLE OF HR VALUES
C----- THE LAST NUMBER OF THE HR AND HS VALUES MUST BE A NEGATIVE
C----- NUMBER. THE REST OF THE FIELD BEING FILLED WITH ZEROS OR
C----- BLANKS AS THE DATA IS READ IN IN GROUPS OF 12.
C----- THE MAXIMUM NUMBER OF HR AND HS VALUES IS 3000 EACH.
C----- THE PROGRAM WILL BE TERMINATED IF THE NO OF VALUES EXCEEDS
C----- 3000.
C----- IF THE FIRST HR OR HS VALUE ON A RECORD IS ZERO, THE WHOLE
C----- RECORD WILL BE IGNORED ON THE ASSUMPTION THAT THE RECORD WAS
C----- EMPTY (I.E. NO CHARACTERS PRECEDED THE CR AND LF).
C
C----- THEREAFTER
C----- THE DATA MUST BE PUNCHED IN THE FOLLOWING ORDER-
C----- IDENTIFICATION OF THE SAMPLE MUST BE PUNCHED IN THE
C----- FOLLOWING WAY-
C----- FIRST          30 CHARACTERS FOR THE SAMPLE NAME.
C----- SECOND         25 FOR THE SOLVENT.
C----- THIRD          8 FOR THE DATE.
C----- FOURTH         3 FOR THE MODE.- 'ORD' OR 'CD'
C----- THE PARAMETERS FOLLOW IN F10.2 FORMAT AND ARE-
C----- FIFTH          MRW - MEAN RESIDUE WEIGHT OF SAMPLE
C----- SIXTH          S - SENSITIVITY SETTING ON INSTRUMENT IN DEG/
C-

```



```

C-----
C
  INTEGER IMODE(2),MODE(3),SAMPL(30),REFER(25),DATE(8),IOVER(3)
  INTEGER HS(3000),HR(3000)
  INTEGER PT
  REAL MRW,S,LEN,CON,WE,FDC,CF,CS,SW,SS,WLSPF,FPV
C----- YY1 = Y-COORDINATES OF POINTS PLOTTED.
C----- YY2 = Y-COORDINATES OF POINTS PLOTTED. (CORRECTED TO SMOOTH)
  DIMENSION YY1(1000),YY2(1000)
  COMMON SW,MODE,SAMPL,REFER,DATE,WL,YY,YMIN,YMAX,XMIN,XMAX,IPEN
  COMMON FW
  EQUIVALENCE (YY1(1),HS(10)),(YY2(1),HR(10))
C---- YMIN,YMAX,XMIN,XMAX MUST BE IN COMMON TO PRESERVE THEIR VALUES
C---- BETWEEN CALLS TO 'PLOT'.
C-----VARIABLES AND PARAMETERS.
C-----
C-----YY=MEAN RESIDUE ROTATION/ELLIPTICITY.
C-----HS=SIGNAL HEIGHT OF SAMPLE.
C-----HR=SIGNAL HEIGHT OF REFERENCE.
C-----MRW=MEAN RESIDUE WEIGHT OF SAMPLE.
C-----S=SENSITIVITY SETTING ON INSTRUMENT IN DEG/CM.
C-----LEN=LENGTH OF CELL IN CM.
C-----CON=CONCENTRATION OF SAMPLE IN MG/ML.
C-----CS=CHART SPEED IN CM/MIN.
C-----WE=WAVELENGTH EXPANSION IN NM/CM.
C-----FDC=FREQUENCY OF DATA COLLECTION. IN SEC.
C-----CF=FREQUENCY AT WHICH CALCULATIONS HAVE TO BE PERFORMED.
C-----SW=STARTING WAVELENGTH.
C-----SS=SCANNING SPEED.
C-----WLSPF=WAVELENGTH SPAN CORRESPONDING TO PUNCH FREQUENCY.
C-----SAMPL=NAME OF THE SAMPLE.
C-----REFER=NAME OF THE REFERENCE.
C-----MODE=ORD OR CD.
C-----WL=WAVELENGTH.
  DATA IMODE(1),IMODE(2) / 'C','D' /
  DATA PT / 4 /
  DATA IBLNK / ' ' /
  CALL RBOUT(33)
C---- TREAT EXCLAMATION MARK AS RUBOUT CHARACTER
C-----READ IN ALL THE INFORMATION OFF PAPER TAPE.
  J=1
  K=12
  1 READ(PT,903)(HS(L),L=J,K)
  IF ( HS(J) ) 5, 1, 2
C---- IF THE FIRST VALUE IS ZERO, ASSUME AN EMPTY RECORD
  2 DO 3 I=J,K
    IF(HS(I))5,5,3
  3 CONTINUE
  J=K+1
  K=K+12
C-----TEST TO ENSURE THAT THERE ARE NO MORE THAN 3000 INPUT VALUES
  IF(K-3000)1,1,4

```

```

C-----IF IT DOES WRITE OUT AN ERROR MESSAGE.
  4 WRITE(3,912)
    STOP
  5 ISTOP=1-1
C----- ISTOP - NUMBER OF POINTS STORED.
    J=1
    K=12
  6 READ(PT,903)(HR(L),L=J,K)
    IF ( HR(J) ) 9, 6, 7
C----- IF THE FIRST VALUE IS ZERO, ASSUME AN EMPTY RECORD
  7 WRITE (3,904) (HS(L),L=J,K), (HR(L),L=J,K)
    IF(K-ISTOP) 8,9,9
  8 J=K+1
    K=K+12
    GO TO 6
C-----READ IN THE IDENTIFICATION OF THE SAMPLE.
  9 READ (PT,900) SAMPL
  900 FORMAT(30A1)
C---- IF HEADING IS ALL BLANK, ASSUME AN EMPTY RECORD
    DO 10 J=1,30
    IF ( SAMPL(J) - IBLNK ) 11, 10, 11
  10 CONTINUE
    GO TO 9
  11 READ (PT,901) REFER, DATE, MODE
  901 FORMAT(25A1/8A1/3A1)
C-----
C-----WRITE OUT A HEADING.
    WRITE(3,902)
    WRITE(3,906)SAMPL,REFER,DATE
C-----READ IN ALL THE PARAMETERS USED.
    READ(PT,905)MRW,S,LEN,CON,WE,FDC,CF,CS,SW
    WRITE(3,907)MODE,MRW,S,LEN,CON,CS,WE,FDC,SW,CF
C-----TEST WHETHER THE MODE IS CD OR ORD.
    DO 12 I=1,3
    IF(IMODE(I)-MODE(I))12,13,12
  12 CONTINUE
C-----WRITE A HEADING.
    WRITE(3,908)
    GO TO 14
  13 WRITE(3,909)
  14 WRITE(3,910)
C-----
C-----PERFORM THE REQUIRED CALCULATIONS AND PLOT THE GRAPH.
C-----
C-----CALCULATE THE SCANNING SPEED.
    SS=CS*WE
C-----CALCULATE THE WAVELENGTH SPAN CORRESPONDING TO THE PUNCH
C-----FREQUENCIES.
    WLSPF=SS*FDC/60.
C-----CALCULATE THE FREQUENCY WITH WHICH THE PUNCHED VALUES ARE
C-----BE USED IN CALCULATIONS.
    FPV=CF/WLSPF

```



```

M=IFIX(FPV+0.5)
C----- RAN = RANGE OF X AXIS.
RAN=((ISTOR-1)/M+1)*CF
FW=SW-RAN
C----- ESTIMATE MAX AND MIN VALUES OF YY.
YMAX=0.
YMIN=0.
JJ=0
DO 18 I=1,ISTOR,M
C-----CALCULATE THE DIFFERENCE BETWEEN HS AND HR AND CONVERT THIS
C-----DIFFERENCE TO CM.
DIFF=FLOAT(HS(I)-HR(I))/25.
Y=(DIFF*MRW*S*100.)/(LEN*CON)
C----- STORE VALUE OF Y IN ARRAY FOR SUBSEQUENT PLOTTING.
JJ=JJ+1
YY1(JJ)=Y
IF(Y-YMIN)15,16,16
15 YMIN=Y
16 IF(Y-YMAX)18,18,17
17 YMAX=Y
18 CONTINUE
YMIN=(IFIX(YMIN)/10-1)*10.
YMAX=(IFIX(YMAX)/10+1)*10.
NPP=JJ
C----- NPP - NUMBER OF POINTS PLOTTED.
C----- PLOT POINTS CALCULATED (ACTUAL)
IVAR=1
WL=SW
DO 19 JJ=1,NPP
YY=YY1(JJ)
WRITE(3,911)WL,YY
CALL PLOT(IVAR)
WL=WL-CF
IVAR=2
19 CONTINUE
C----- SMOOTH POINTS USING 5-POINT QUADRATIC FIT
C----- REPEATED A NUMBER OF TIMES TO IMPROVE THE APPEARANCE.
DO 22 KSM=1,5
NT=NPP-2
DO 20 JJ=3,NT
YY2(JJ)=(3.4*YY1(JJ) + 2.4*(YY1(JJ-1)+YY1(JJ+1))
$ - 0.6*(YY1(JJ-2)+YY1(JJ+2)))/7.
20 CONTINUE
C----- POINTS 1 AND 2
A=(2.0*(YY1(5)+YY1(1))-(YY1(4)+YY1(2))-2.*YY1(3))/14.
B=(2.0*(YY1(5)-YY1(1))+(YY1(4)-YY1(2)))/10.
YY2(1)=4.0*A-2.*B+YY2(3)
YY2(2)= A-B+YY2(3)
C----- POINTS NPP AND NPP-1
A=(2.0*(YY1(NPP)+YY1(NPP-4))-(YY1(NPP-1)+YY1(NPP-3))
$ -2.0*YY1(NPP-2))/14.
B=(2.0*(YY1(NPP)-YY1(NPP-4))+(YY1(NPP-1)-YY1(NPP-3)))/10.

```



```

YY2(NPP)=4.*A+2.*B+YY2(NPP-2)
YY2(NPP-1)= A+B+YY2(NPP-2)
DO 21 JJ=1,NPP
YY1(JJ)=YY2(JJ)
21 CONTINUE
22 CONTINUE
WL=SW
IVAR=4
DO 23 JJ=1,NPP
YY=YY1(JJ)
CALL PLOT(IVAR)
WL=WL-CF
23 CONTINUE
CALL PLOT ( 3, INT, IOVER )
STOP
902 FORMAT(1H0,40X,19HORD/CD CALCULATIONS/12H0INPUT DATA-)
903 FORMAT(3X,12(1X,14))
904 FORMAT(1X,14,11I5,2H *,14,11I5)
905 FORMAT(F10.3)
906 FORMAT(/16H IDENTIFICATION-/1X,16X,6HSAMPLE,26X,7HSOLVENT,25X,4HDS
  $ATE/17X,30A1,2X,25A1,7X,8A1)
907 FORMAT(1X,5HMODE-/1X,16X,3A1/1X,11HPARAMETERS-/1X,16X,4HMRW=,F6.2
  $1X,2HS=,F5.3,1X,4HLEN=,F5.2,1X,4HCON=,F5.3,1X,3HCS=,F5.2,1X,3HWE=,
  $F5.2,1X,4HFDC=,F5.2,1X,3HSHW=,F6.2,1X,3HCF=,F5.2//)
908 FORMAT(1X,(10HWAVELENGTH,2X,21HMEAN RESIDUE ROTATION,6X))
909 FORMAT(1X,10HWAVELENGTH,2X,24HMEAN RESIDUE ELLIPTICITY,3X)
910 FORMAT(4X,4H(NM),8X,18H(DEG.SQ.CM./DMOLE))
911 FORMAT(1X,F7.2,F20.2)
912 FORMAT(1X,35HNUMBER OF INPUT VALUES EXCEEDS 3000/)
END

```

C***** SUBROUTINE TO PLOT *****

```

C
      SUBROUTINE PLOT(IVAR)
C----- IVAR = 1 - PLOTS OUTLINE, AXIS ETC. FOLLOWED BY 1ST POINT.
C-----      = 2 - PLOTS A POINT.
C-----      = 3 - TERMINATES THE PLOTTER.
C-----      = 4 - PLOTS THE BEST LINE THROUGH THE POINTS.
      INTEGER IMODE(2),MODE(3),SAMPL(30),REFER(25),DATE(8)
      COMMON SW,MODE,SAMPL,REFER,DATE,X,Y,YMIN,YMAX,XMIN,XMAX,IPEN
      COMMON FW
C- YMIN,YMAX,XMIN,XMAX MUST BE IN COMMON TO PRESERVE THEIR VALUE
      DATA IMODE(1),IMODE(2) / 'C','D' /
      DATA PI / 3.1415927 /
C
      GO TO (1,8,9,10),IVAR
C-----INITIALISE THE SIZE OF THE RECTANGLE ENCLOSING THE GRAPH.
      1 CALL FSIZE(16,20)
C-----INITIALISE THE ORIGIN
C-----CALCULATE THE SCALE ALONG THE X AND Y AXIS
      SCAL=(IFIX(YMAX-YMIN)+24)/25
      YS=1./SCAL*17./25.
      XSCAL=(IFIX(SW-FW)+15)/16
      XS=1./XSCAL*13./16.
      XO=SW-18.*XSCAL
      YO=YMIN-2.*SCAL
      CALL FSCAL(XS,YS,XO,YO)
C-----SET UP THE X-AXIS.
      XGO=SW-XSCAL*16.
      YGO=0.
      DIST=XSCAL
      LNTH=16
      CALL FGRID(0,XGO,YGO,DIST,LNTH)
C-----INSERT THE X VALUES.
      XSTRT=SW
      YSTRT=YGO
      XWDTH=0.15
      YHGHT=0.2
      ANGLE=3.*PI/2.
      DO 2 I=1,4
      CALL FCHAR(XSTRT,YSTRT,XWDTH,YHGHT,ANGLE)
      WRITE(7,900)XSTRT
900 FORMAT(F5.0)
      XSTRT=XSTRT-4.*DIST

```


C-----SET UP THE Y-AXIS AND INSERT THE Y VALUES.

XSTRT=XGO-1.5*XSCAL

YSTRT=YMIN

ANGLE=0.

DO 3 I=1,26

CALL FCHAR(XSTRT,YSTRT,XWIDTH,YHGT,ANGLE)

WRITE(7,901)YSTRT

901 FORMAT(F7.0)

YSTRT=YSTRT+SCAL

3 CONTINUE

YSTRT=YMIN+25.*SCAL

DIST=SCAL

LNTH=25

CALL FGRID(3,XGO,YSTRT,DIST,LNTH)

C-----INITIALISE FOR THE GRAPH LABELS.

XSTRT=SW-XSCAL*17.

YSTRT=YMIN-SCAL*1.5

XWIDTH=0.15

YHGT=0.24

ANGLE=0.

CALL FCHAR(XSTRT,YSTRT,XWIDTH,YHGT,ANGLE)

WRITE(7,902)SAMPL,REFER,DATE

902 FORMAT(7HSAMPLE-,30A1,2X,8HSOLVENT-,25A1,2X,5HDATE-,8A1)

XSTRT=FW+2.*XSCAL

YSTRT=YMAX+SCAL/2.

CALL FCHAR(XSTRT,YSTRT,XWIDTH,YHGT,ANGLE)

C-----TEST WHETHER THE MODE IS CD OR ORD.

DO 4 I=1,3

IF(1MODE(I)-MODE(I))4,5,4

4 CONTINUE

GO TO 6

5 WRITE(7,903)

903 FORMAT(39HGRAPH OF ELLIPTICITY AGAINST WAVELENGTH)

GO TO 7

6 WRITE(7,904)

904 FORMAT(44HGRAPH OF OPTICAL ROTATION AGAINST WAVELENGTH)

C-----

7 IPEN=1

C-----

C-----PLOT A POINT.

C-----

8 CALL FPLLOT(IPEN,X,Y)

CALL POINT(2)

IPEN=1

RETURN

C----- TERMINATE - RAISE PEN AND RETURN TO ORIGIN

9 CALL FPLLOT(1,XO,YO)

C----- DRAWS A X AT BOTTOM CORNER OF PAGE TO CHECK PLOTTER ACCURACY.

CALL POINT(2)

CALL FPLLOT(1,XO,YO)

RETURN

C-----PLOT THE SMOOTH LINE.

10 CALL FPLLOT(IPEN,X,Y)

IPEN=2

RETURN

END

APPENDIX 4

Circular dichroism spectra of normal IgM, IgM(Sad) and some of their tryptic fragments and reduced and alkylated subunits and polypeptides.

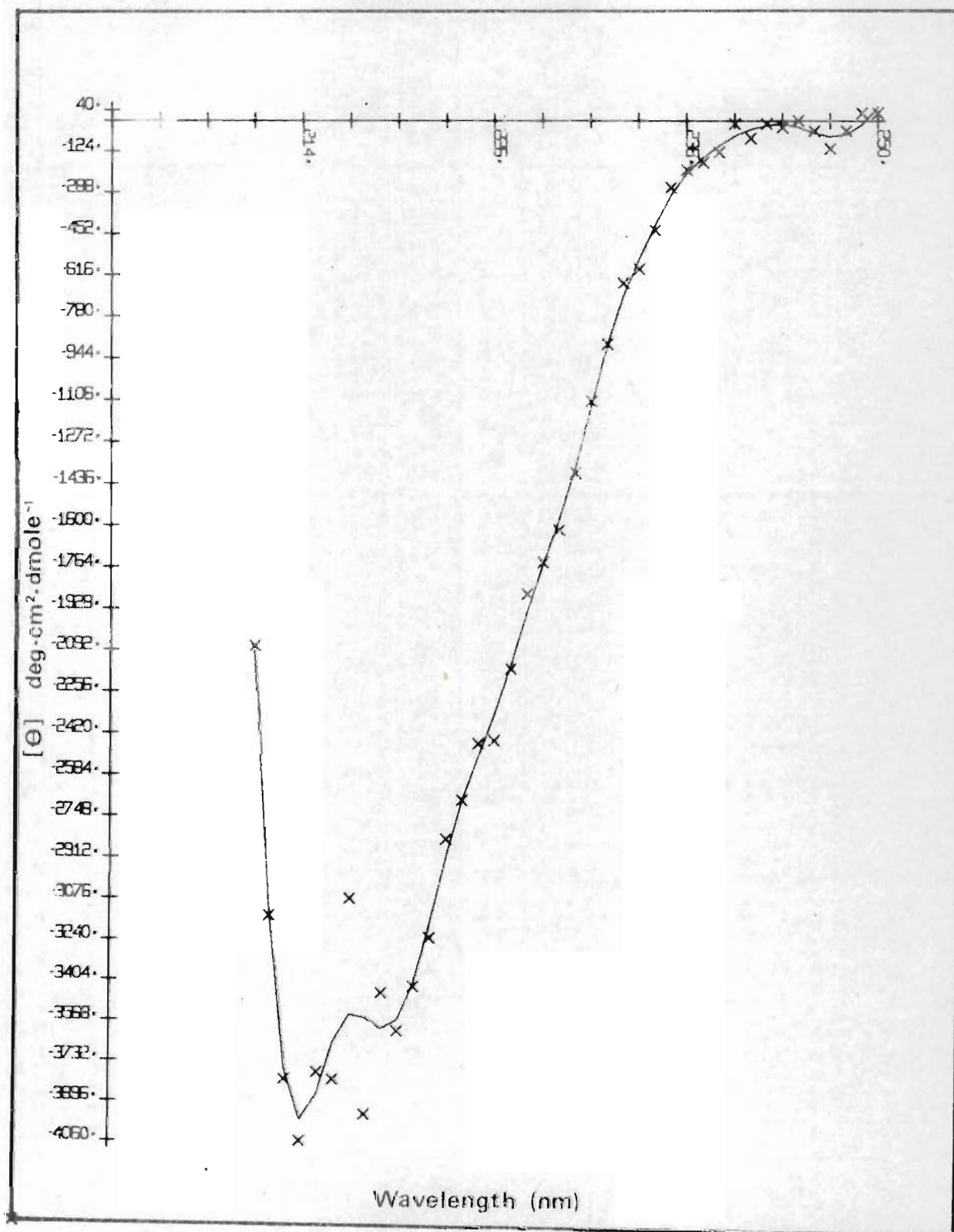


Fig. A1: CD spectrum (205-250 nm) of normal IgM (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 1.54 mg/ml)

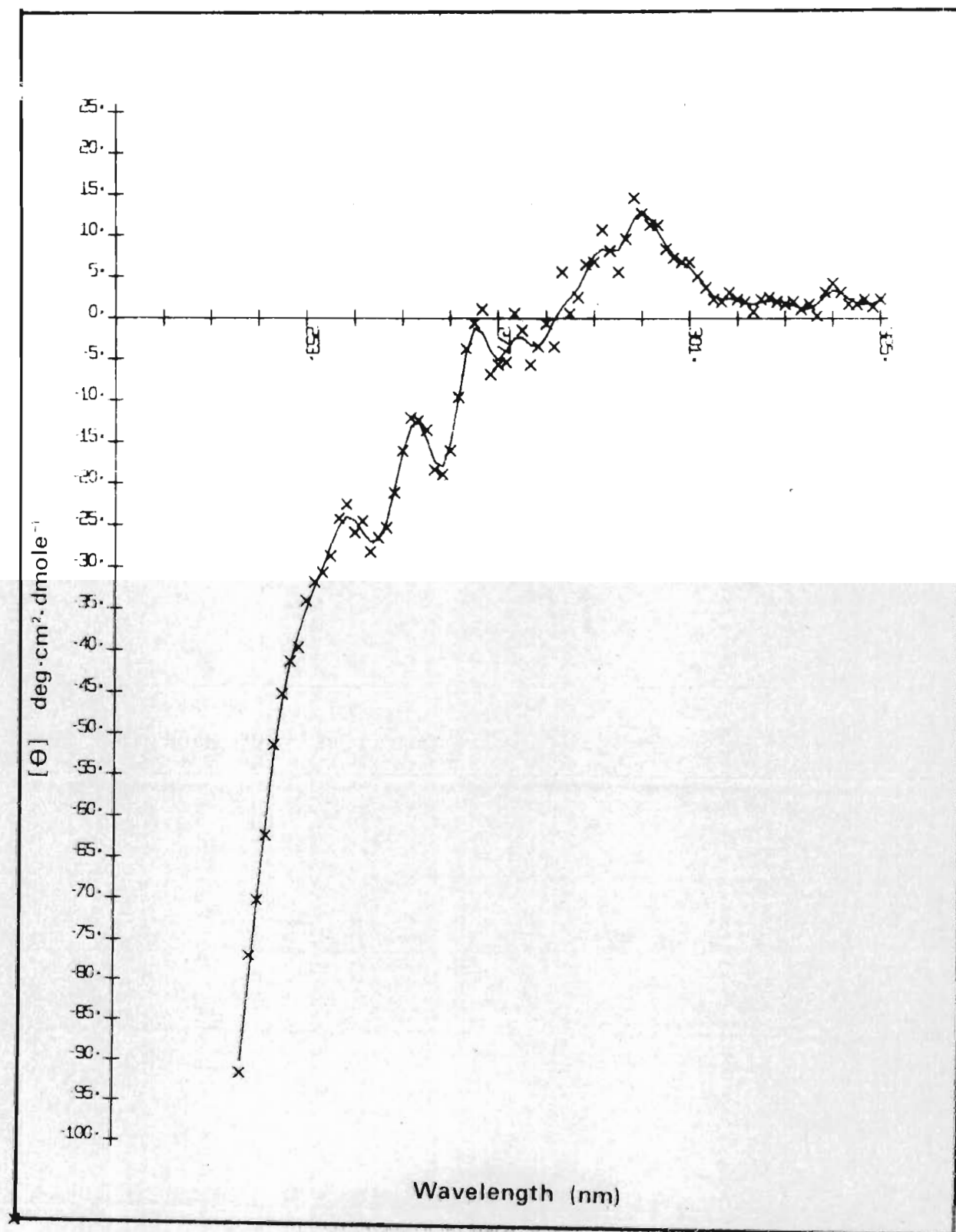


Fig. A2: CD spectrum (235-325 nm) of normal IgM (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 1.54 mg/ml)

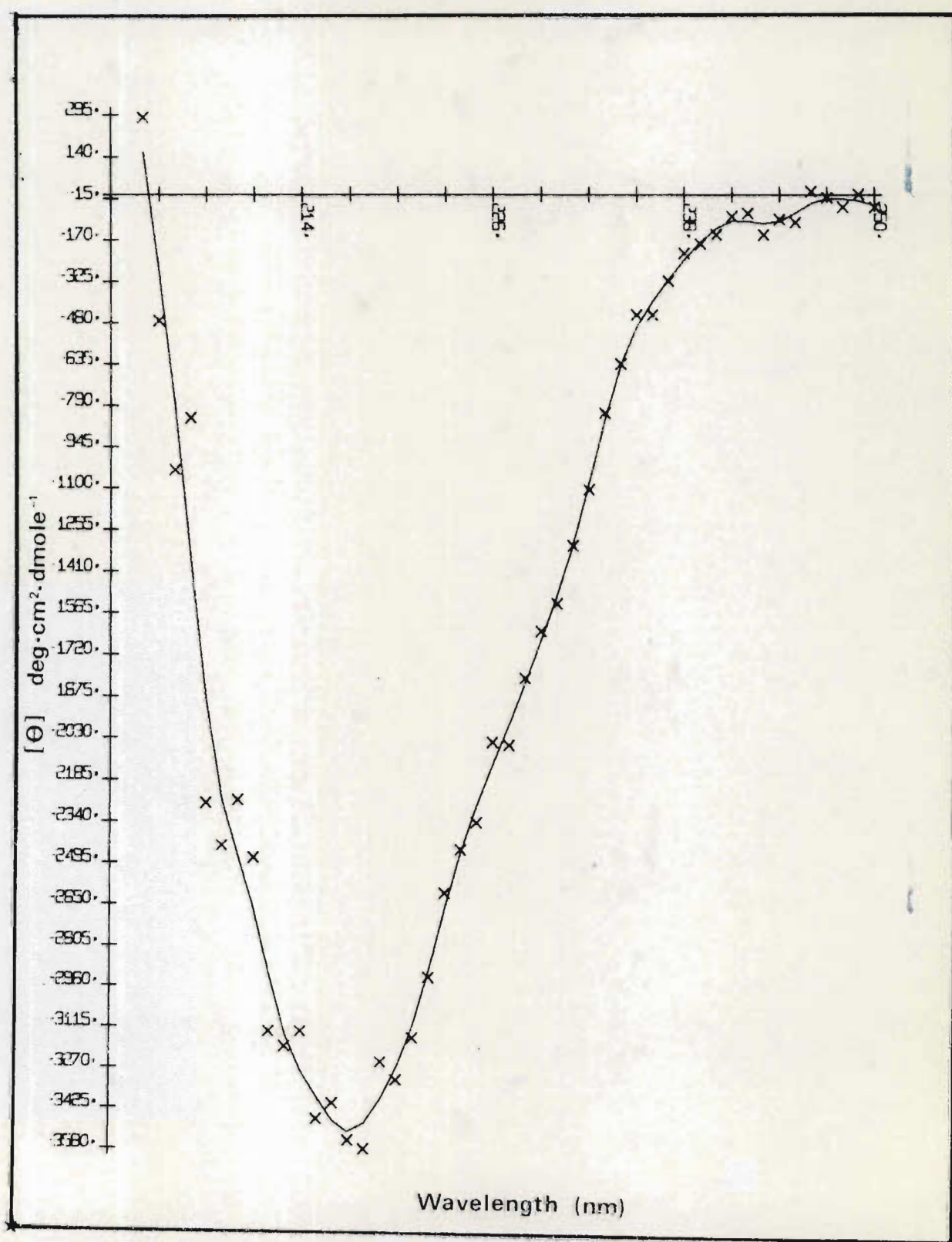


Fig. A3: CD spectrum (205-250 nm) of 7-S normal IgM (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.754 mg/ml)

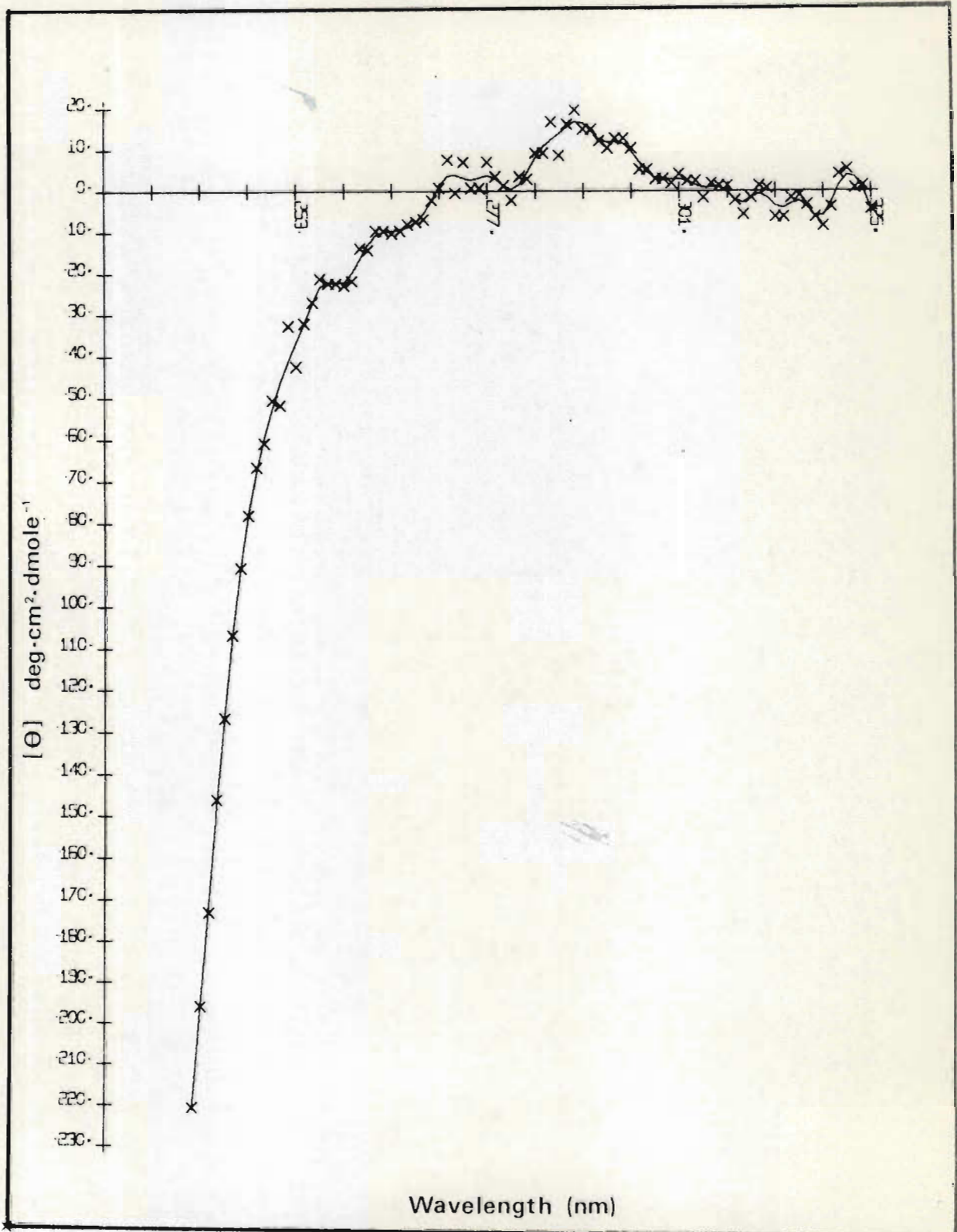


Fig. A4: CD spectrum (235-325 nm) of 7-S normal IgM (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.754 mg/ml)

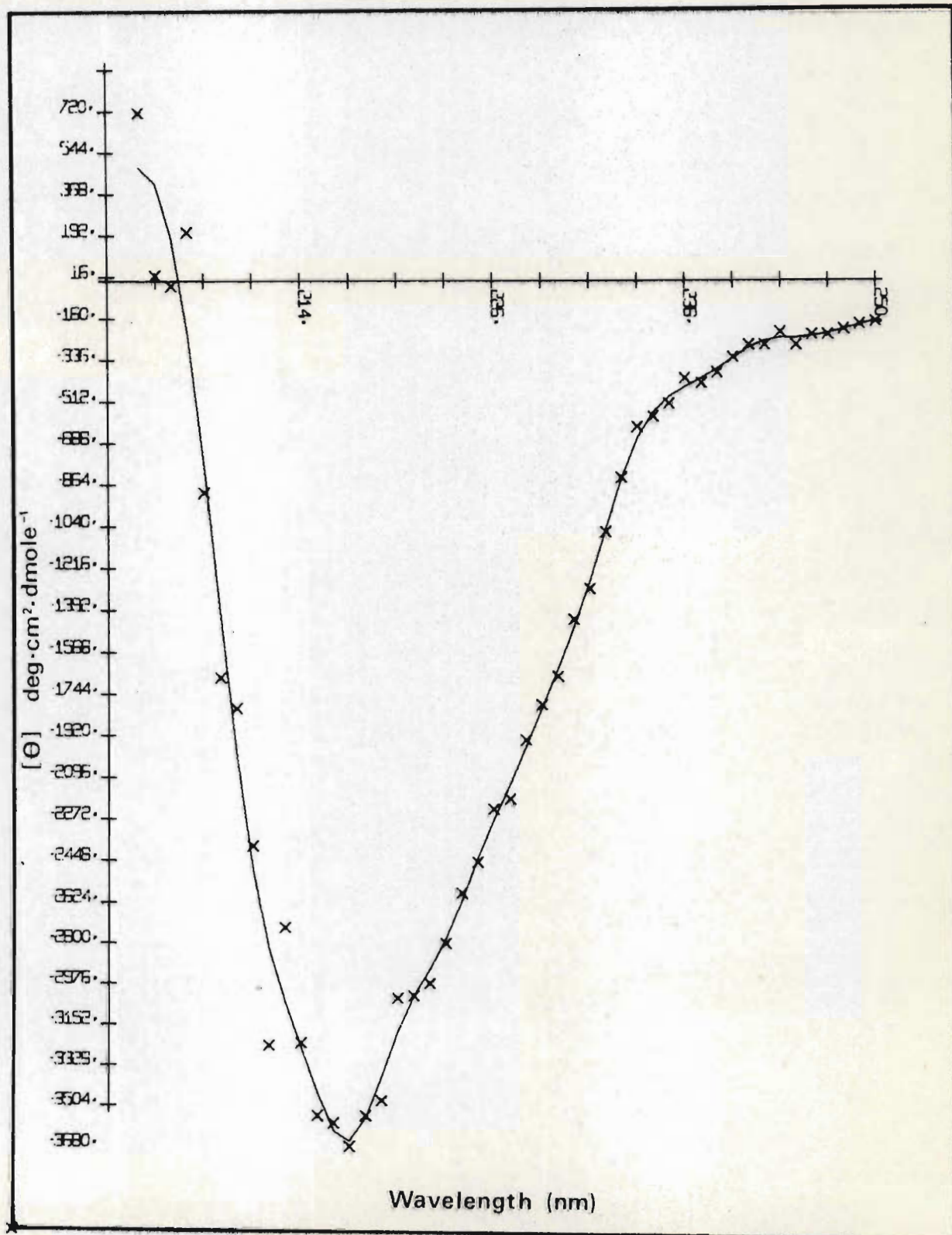


Fig. A5: CD spectrum (205-250 nm) of 7-S IgM(Sad) (buffer, 0.075 M-phosphate, pH 7.0; cell 0.1 cm; concentration, 0.800 mg/ml)

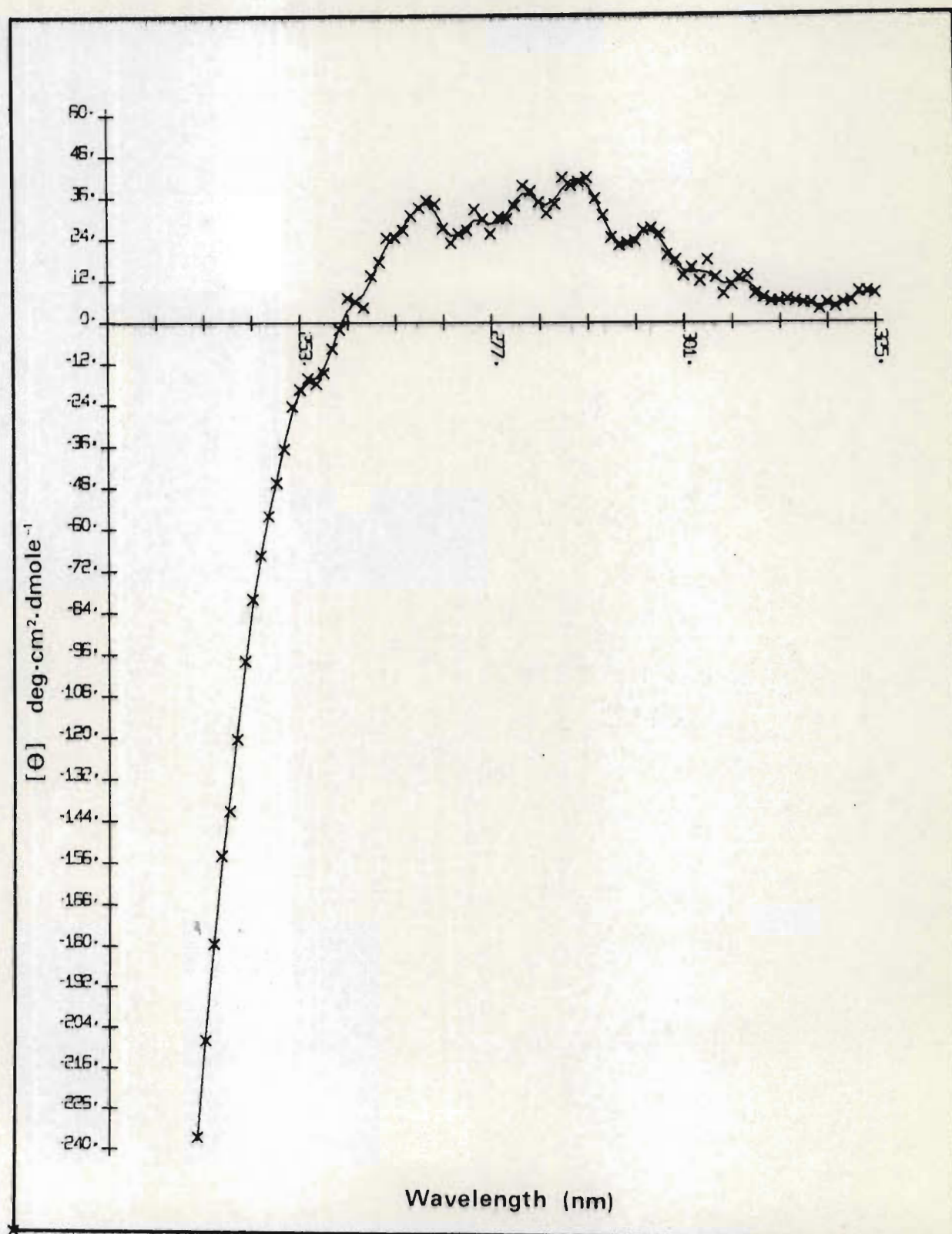


Fig. A6: CD spectrum (235-325 nm) of 7-S IgM(Sad) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.800 mg/ml)

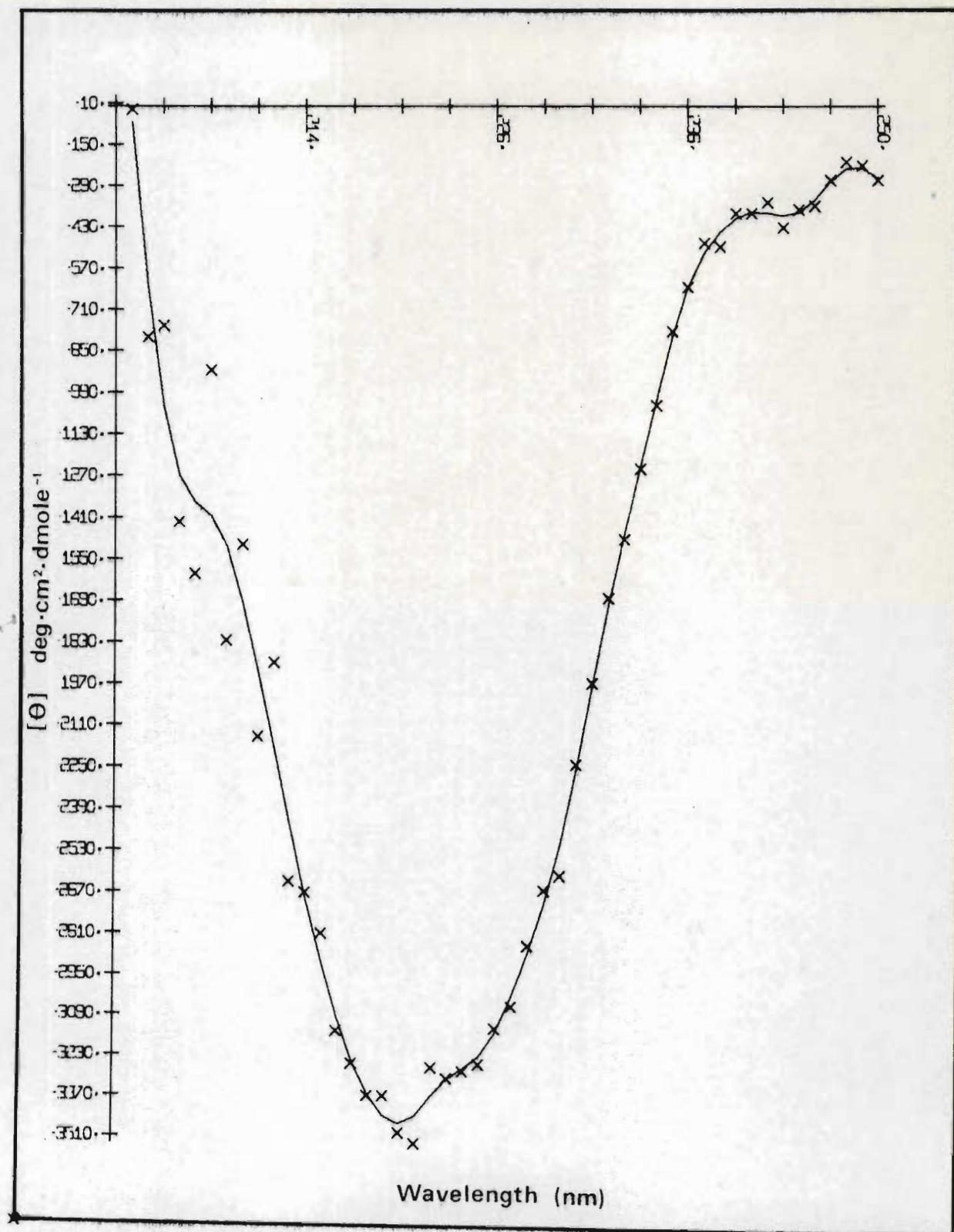


Fig. A7: CD spectrum (205-250 nm) of $\text{Fc}\mu(\text{Sad})$ (fraction 25b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.695 mg/ml)

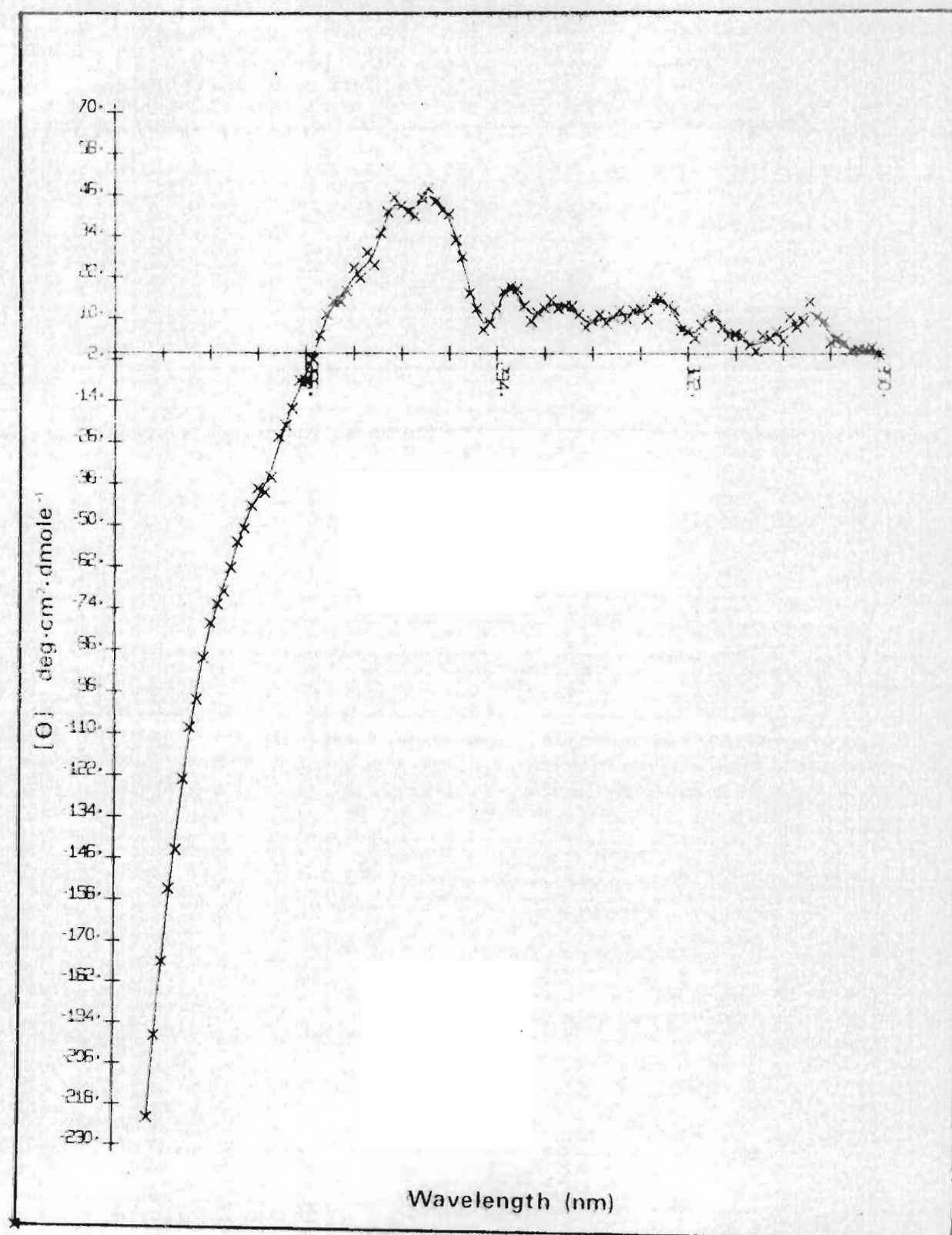
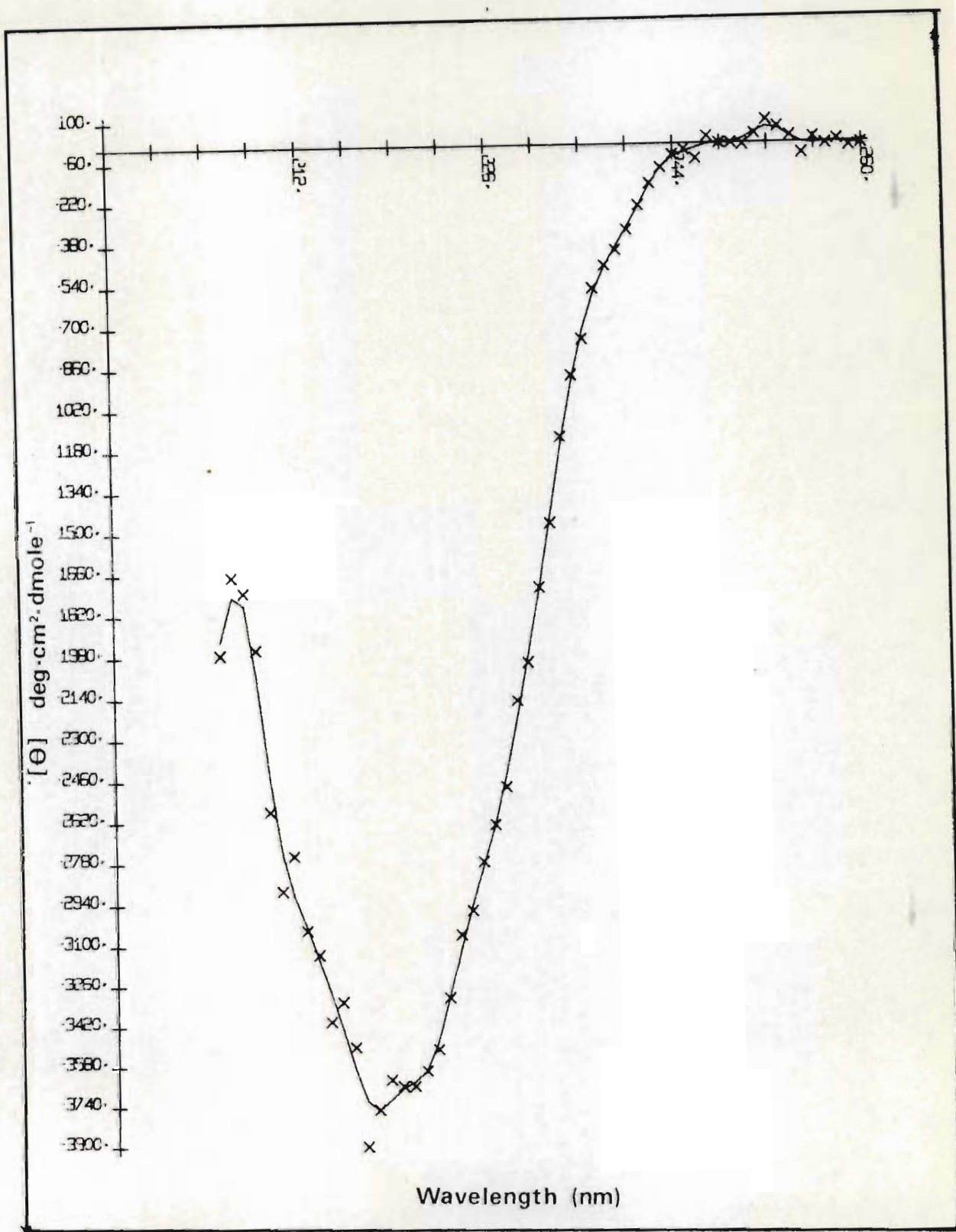


Fig. A8: CD spectrum (245-350 nm) of Fcμ(Sad) (fraction 24b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.695 mg/ml)



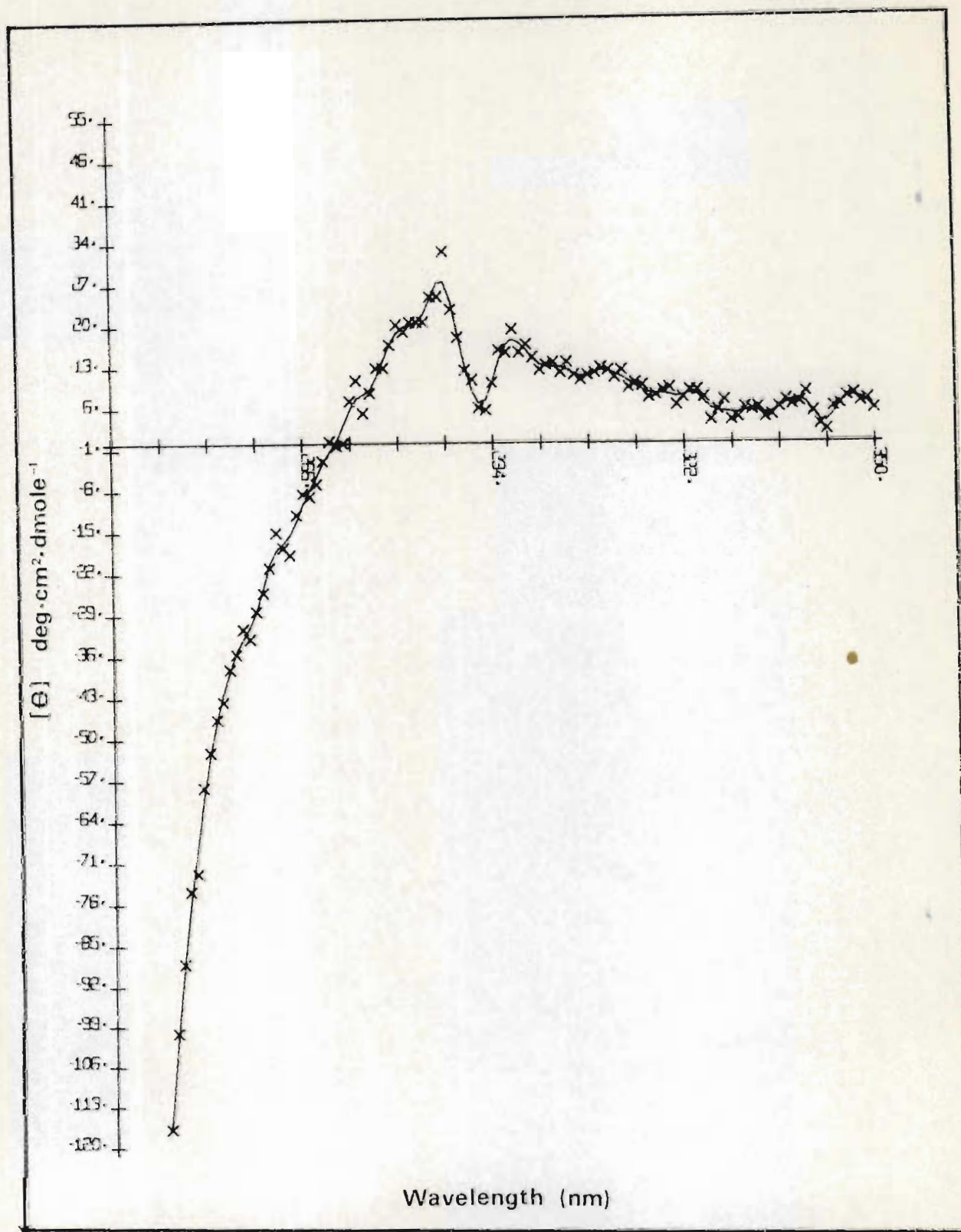


Fig. A10: CD spectrum (245-350 nm) of normal Fc₅u (fraction 15b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 1.010 mg/ml)

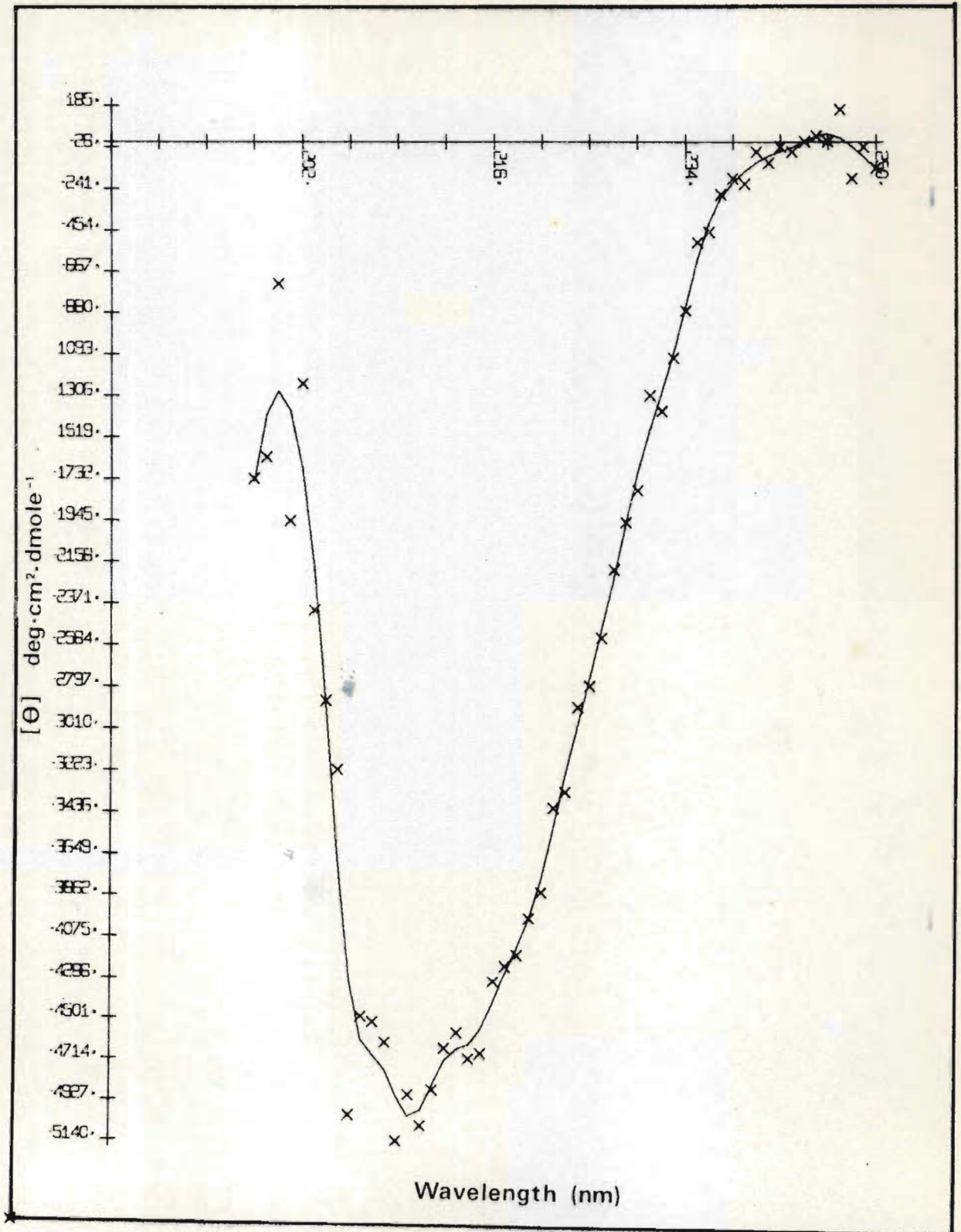


Fig. A11: CD spectrum (190-250 nm) of normal Fc_{5u} after renaturation from 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0 (fraction 16b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.320 mg/ml)

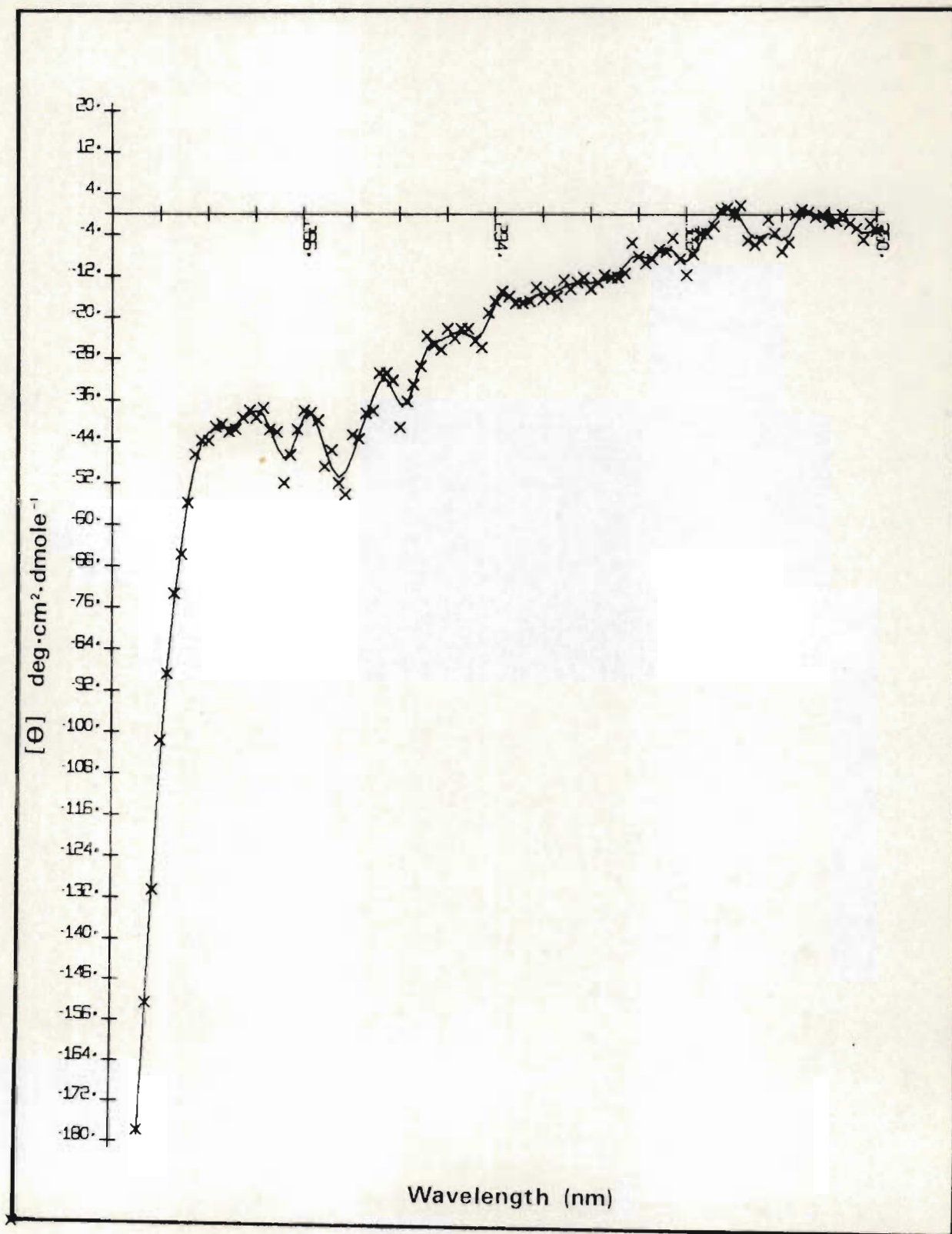


Fig. A12: CD spectrum (245-350 nm) of normal $\text{Fc}_5\mu$ after renaturation from 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0 (fraction 16b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.960 mg/ml)

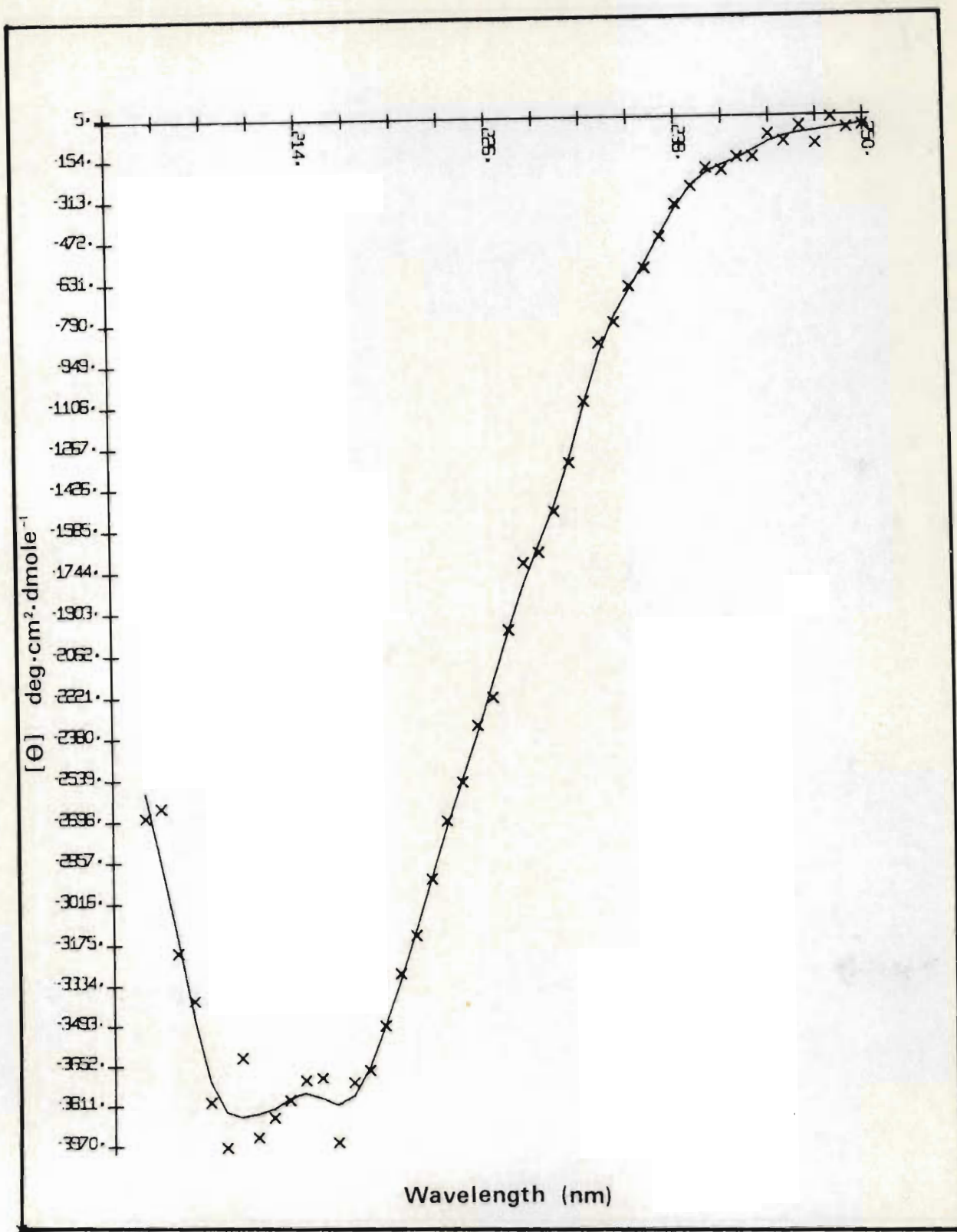


Fig. A13: CD spectrum (205-250 nm) of $\text{Fc}_{5\mu}(\text{Sad})$ after renaturation from 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0 (fraction 20b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.880 mg/ml)

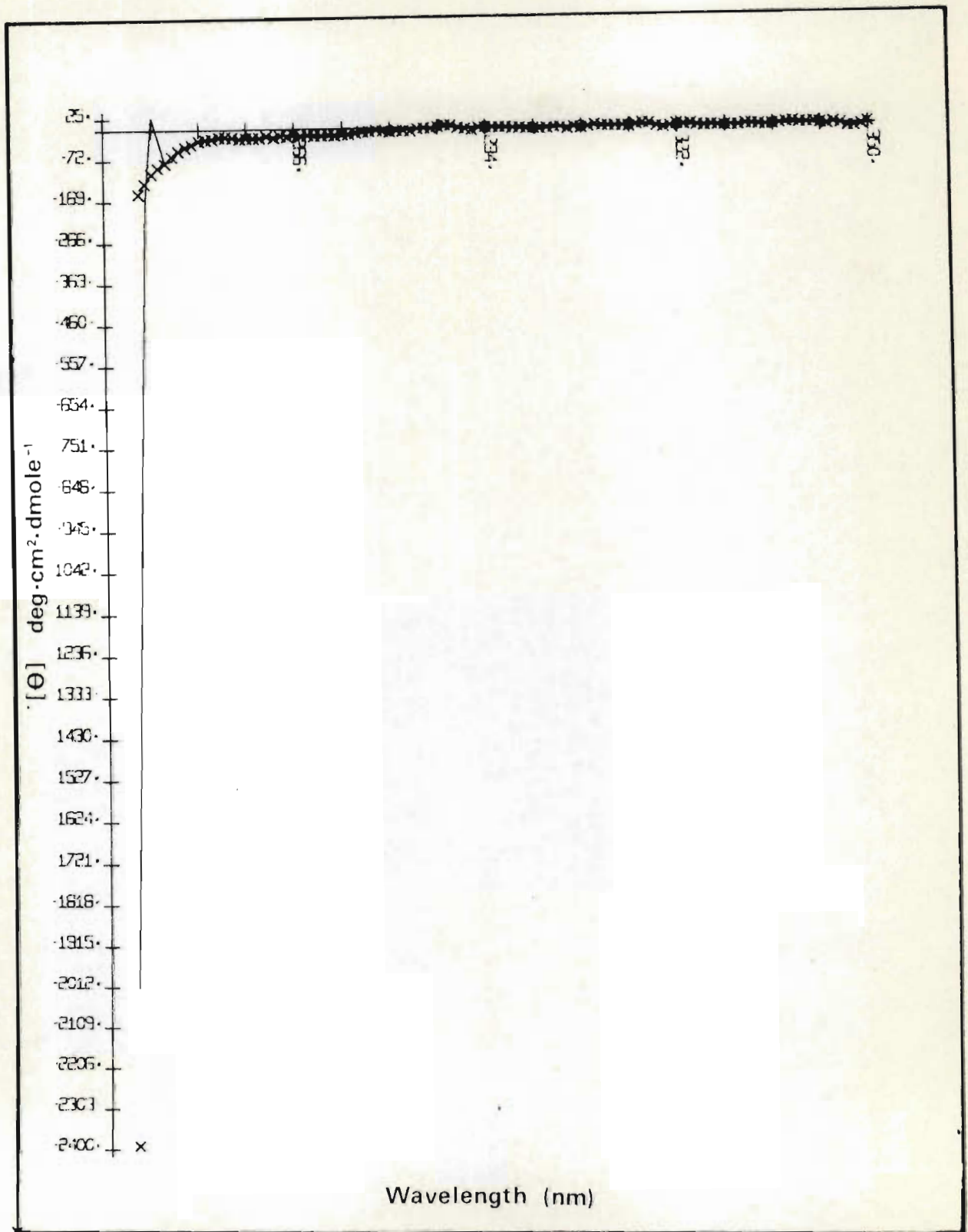


Fig. A14: CD spectrum (245-350 nm) of $\text{Fc}_5\text{u}(\text{Sad})$ after renaturation from 0.01 M-Tris-4.0 M-GuHCl buffer, pH 8.0 (fraction 20b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.880 mg/ml)

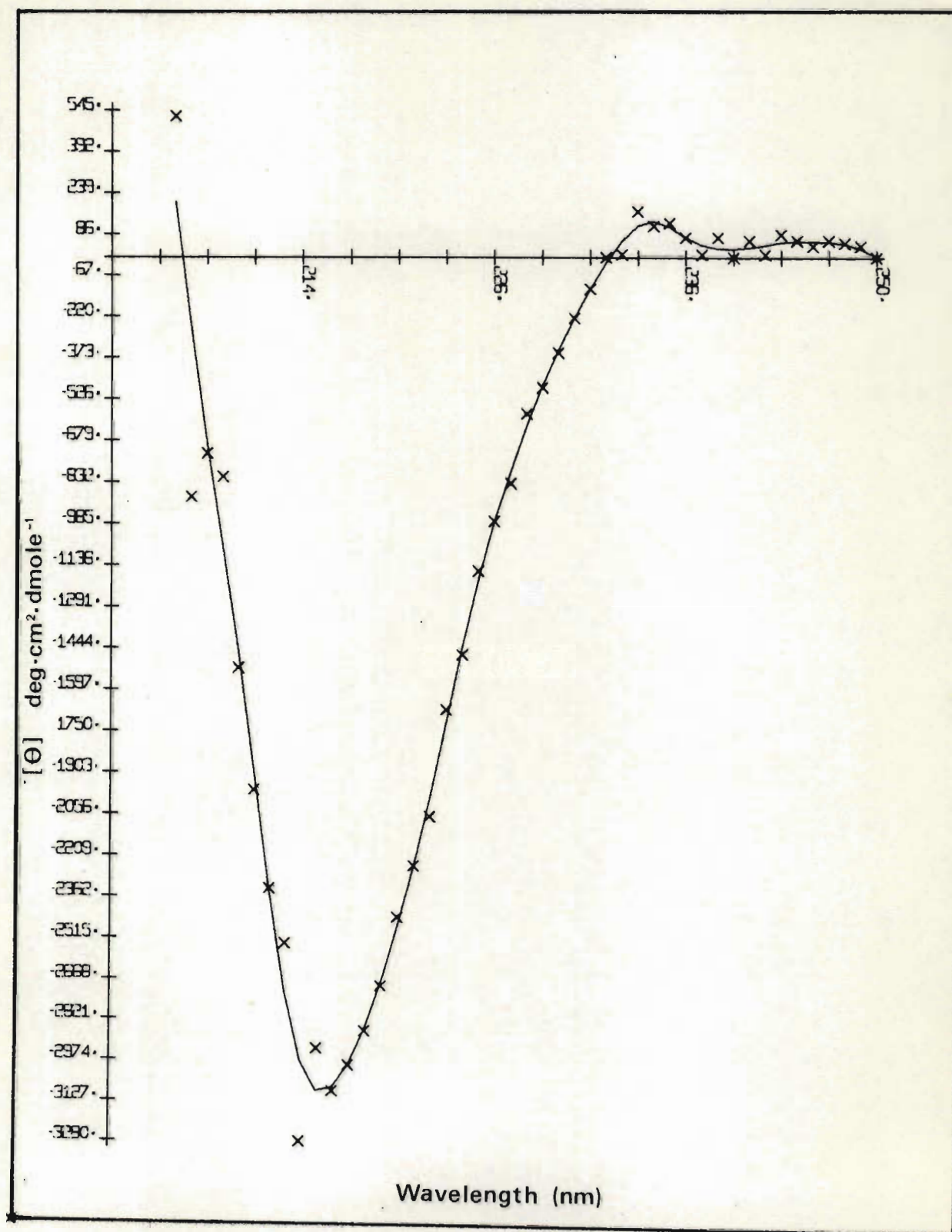


Fig. A15: CD spectrum (205-250 nm) of normal Fab μ (fraction 14b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 0.1 cm; concentration, 0.809 mg/ml)

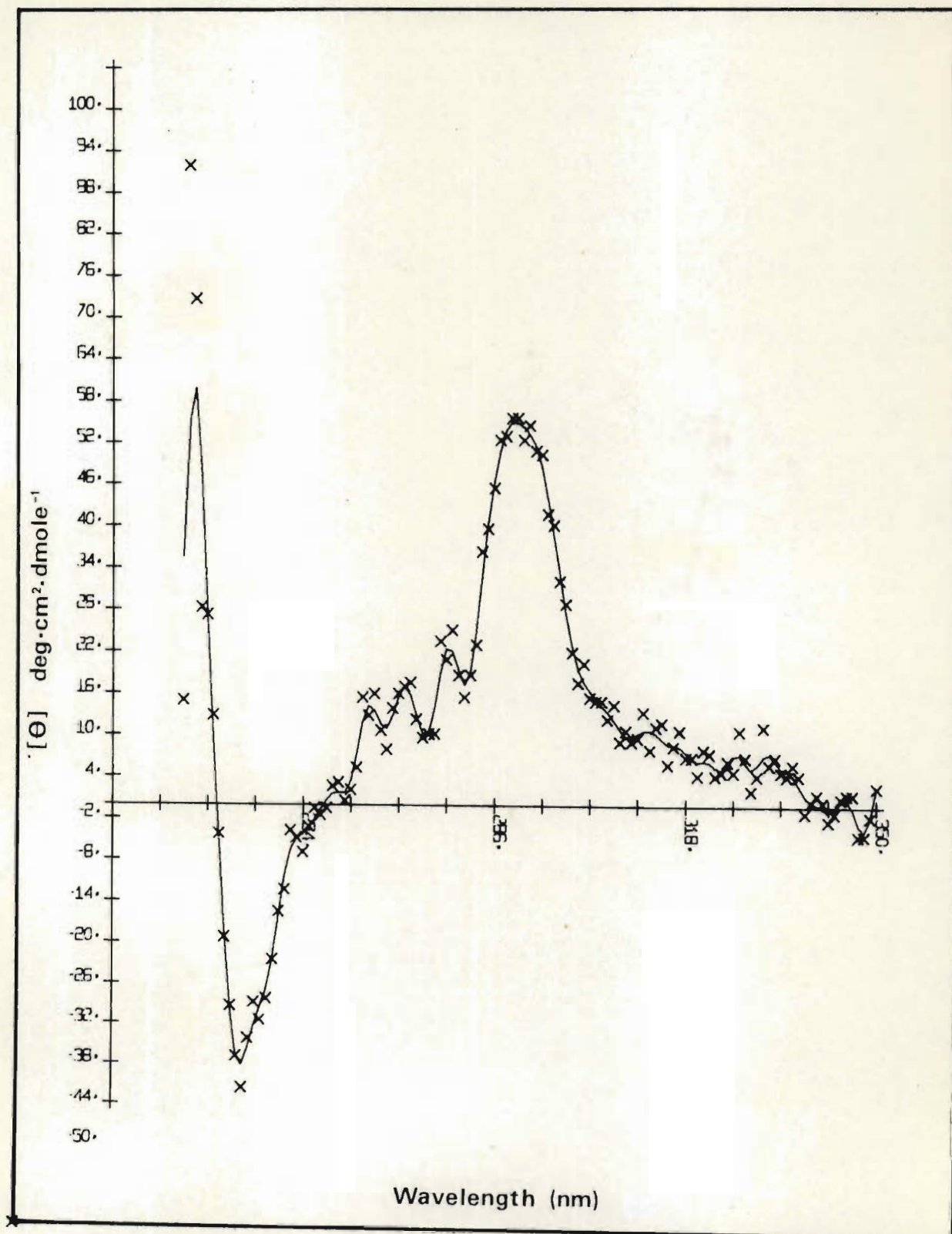


Fig. A16: CD spectrum (230-350 nm) of normal Fab μ (fraction 14b) (buffer, 0.075 M-phosphate, pH 7.0; cell, 1.0 cm; concentration, 0.809 mg/ml)